BIO-CHEMICAL RESEARCHES,

A THESIS SUBMITTED FOR THE DEGREE OF D.Sc.

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PREFACE.

The first two papers comprising this Thesis represent work done in the Bio-Chemistry Department, Liverpool University.

The rest of the work has been carried out in the Physiology Department, Aberdeen University.

I wish to record my gratitude to Prof. B. Moore, F.R.S. and to Prof. J.A.MacWilliam, F.R.S. for much kindness and encouragement received in these laboratories respectively.

All the work on which these papers are based has been done entirely by myself, and the thesis has also been composed by myself.

Physiology Department,
Aberdeen University,
22nd April, 1921.

CONTENTS.

ON THE ACTION OF CHLOROFORM ON THE PROTEINS OF SERUM AND UPON SOLUTIONS OF HAEMOGLOBIN.

(Originally published in the Thompson-Yates and Johnston Laboratories Reports (Liverpool University) Vol. 6, Part 1, 1905).

In a research recently published Moore and Roaf (1) have drawn attention to the formation of compounds between chloroform and the proteins of the blood, and pointed out that even before precipitation occurs there is evidence of the existence of such compounds, which at that stage are unstable in character and depend upon the pressure of chloroform in the blood.

In the present paper the matter has been followed up more completely from the chemical point of view, and a more extended examination made of the precipitate as to its stability and the amount of chloroform it contains. The condition of the protein prior to the stage of precipitation by chloroform alone has also been studied by precipitating the protein with neutral salt, in this case by saturation with ammonium sulphate.

The precipitation of protein from serum or solutions of haemoglobin was noticed first by Salkowski (2), who used chloroform as a preservative for these fluids, and it was also noticed by Horbaczewski (3) and Formanek (4), the latter of whom investigated the matter at some length. He found no chlorine in the precipitate after drying it at 130° C. but the experiments described in this paper would seem to show that any chloroform in an unstable form of combination must be driven off at that temperature, as a more or less constant proportion of chlorine has always been found in the precipitate.

In these experiments the solutions of any desired strength of chloroform were made either by weighing the chloroform into a tared flask by dropping it from a fine capillary pipette, or by

adding an accurately measured volume of pure chloroform to a known volume of the protein solution.

If a solution of haemoglobin is taken, one per cent of chloroform added and the mixture shaken thoroughly, all the chloroform dissolves and no precipitate forms on standing either at room or body temperature. If the percentage of chloroform exceed one and a half a precipitate is formed more or less readily according to the concentration of the chloroform, and also to the temperature, the precipitate being formed more readily at about 37° C. than at room temperature.

The highest concentration of chloroform in these experiments with haemoglobin solutions was 7·5 per cent. In this case a copious brick-red precipitate came down, mixed with a little undissolved chloroform. Before this precipitation occurs, the solution also changes colour and becomes opaque. The precipitate was removed by centrifugalising, and was washed several times with distilled water. On first centrifugalising, the precipitate settled down very rapidly, leaving the fluid above clear and practically colourless. This fluid was tested for protein but only a trace was found, showing that the above concentration of chloroform is sufficient to remove all the protein from solution.

The nature of this precipitate was next investigated. It was thought that the protein was perhaps simply thrown out of solution by the chloroform in an unchanged form, and experiments were carried out with a view to finding whether the protein could be got into solution again.

After the precipitate had been thoroughly washed, to get rid, as far as possible, of any mechanically adherent chloroform, it was warmed to about 40° C. with distilled water under the reduced pressure of a water pump. Even at the ordinary temperature a considerable volume of gas was seen to bubble off, and on warming it came off very vigorously for a time. When there was no further appearance of any gas coming off the mixture of liquid and precipitate was filtered. The filtrate showed the spectrum of oxy-haemoglobin. This led to the idea that a considerable quantity, or perhaps the whole of the precipitate might be made to redissolve, and the rest of it was thoroughly shaken up with water and the liquid examined but no further action had taken place. This experiment was repeated several times, the various operations being carried through as rapidly as possible, but no further success was obtained in the efforts to get the protein into solution again after pumping off the chloroform. The precipitate obtained on adding excess of chloroform to haemoglobin solution, after being washed several times, was found to dissolve easily in sodium hydrate and also in a dilute solution of sodium carbonate, in the latter case giving the spectrum of alkaline haematin.

In these earlier experiments it was assumed that the gas which came off on warming the precipitate with water was chloroform, or at least partly so, but no other method was adopted to prove the presence of or estimate the quantity of chloroform in the

precipitate, and the experiments were for a time abandoned. After a short time, however, a method was tried, after being tested on aqueous solutions of chloroform of known strength. The method consists in warming the substance under examination on a steam bath with a saturated solution of potassium permanganate in fifteen per cent sodium hydrate, both of these reagents being free from chlorine. The chloroform thus gets converted into hydrochloric acid, forming sodium chloride. About fifteen to twenty grammes of the moist precipitate are taken and warmed with from 150 to 200 c.c. of the permanganate solution in a large beaker, the precipitate being mixed up as thoroughly as possible with the liquid by frequent stirring. After warming the precipitate with this solution for a few hours the process is complete. The insoluble matter is now filtered off and washed, and the hydrochloric acid estimated in the filtrate after neutralising with nitric acid.

In the case of haemoglobin solutions, when the amount of chloroform added was insufficient to cause a precipitate, the protein was thrown out of solution by saturation with ammonium sulphate. The precipitate was then filtered off and washed with a saturated solution of that salt, after which it was treated with the alkaline permanganate solution and the hydrochloric acid estimated in the usual way.

In the case of serum, obtained by centrifugalising the blood after defibrination, it was found that it would take up about five per cent of chloroform. The serum becomes very opalescent,

even when considerably smaller percentages of chloroform are added. On centrifugalising after saturation of the serum with chloroform, no precipitate settled out even after three-quarters of an hour, the fluid remaining practically as opalescent as at first. If more chloroform was added than the serum could take up, on centrifugalising, the excess of chloroform was precipitated mixed with only a very small quantity of protein. It was therefore found necessary, in the experiments with serum, after allowing it to take up as much chloroform as possible, to precipitate the protein by saturation with ammonium sulphate. Before doing so, however, the undissolved chloroform was removed by centrifugalising. The precipitated protein after being filtered off was washed with a saturated solution of ammonium sulphate and the chlorine determined in the same way as in the precipitate from haemoglobin.

The following table shows some of the chief results in the case of haemoglobin solutions:-

Percentage of haemoglobin in solution	Percentage of chloroform added	Percentage of chloroform in precipitate to haemoglobin in solution
13·6	0·5	1·55
11·5	1·0	1·60
12·3	2·0	1·30
11·9	3·0	1·70
15·4	7·5	2·20

The high percentage of chloroform in the precipitate in the last result is doubtless due to the difficulty in washing away all the excess of chloroform. In the other experiments all the chloroform would be taken up, Moore and Roaf finding that solutions of haemoglobin will take up over six per cent by weight of chloroform.

These results, considering the nature of the precipitate, seem to show that the amount of chloroform taken up in precipitating the protein from haemoglobin solutions is constant, even though widely different percentages of chloroform are added, and therefore, apart from other facts, it would seem that the precipitate obtained on adding chloroform to solutions of haemoglobin is a definite compound or at least a physical aggregate and not a mere mixture of chloroform and haemoglobin.

The experiments with serum are more troublesome to carry out, owing to the necessity of saturation with ammonium sulphate and the more thorough washing required in order to remove the sodium chloride. The results, however, again point to a more or less constant proportion of chloroform in the precipitated protein.

Percentage of chloroform added to serum	Percentage of chloroform in precipitated protein to protein in the serum.
0·5	2·4
1·0	3·6
2·0	10·9
3·0	12·3
5·0	12·0
6·0	13·5
8·0	16·0

These figures show that the protein is not fully saturated with chloroform until the amount of the latter added is between two and three per cent, the amount of chloroform in the precipitate with lower percentages being approximately proportional to the amount of chloroform added to the serum. As serum takes up only about five per cent of chloroform, the results with six and eight per cent are probably too high, owing to the difficulty in washing away all the adherent chloroform.

An interesting fact was noticed bearing on the stability of the compound between chloroform and haemoglobin. If the latter solution is first saturated with carbon monoxide and then 7·5 per cent of chloroform is added, the solution retains the colour of carbon monoxide haemoglobin and only a very small precipitate, if any, is formed. If say four per cent of chloroform is added no precipitate appears even on standing for several hours. This seems to prove that chloroform forms

a compound which is less stable than carbon monoxide haemoglobin, or at least is not formed in presence of excess of carbon monoxide.

REFERENCES TO LITERATURE.

(1) Moore and Roaf, Proc. Royal Soc. 73, p. 382.

(2) Salkowski, Deutsche med. Wochensch. 1888, No. 16.

(3) Horbaczewski, quoted by Formanek.

(4) Formanek, Zeitsch. f. physiol. Chem. 29, p. 416.

ON GLYCOSURIA

CAUSED BY EXCESS OF CARBON DIOXIDE IN THE AIR BREATHED.

(The main results in this paper were published in the Bio-Chemical Journal, Vol. 1, p. 455).

INTRODUCTION.

The effects of respiration under low pressures of oxygen have been studied for many years, but at first no reference seems to have been made to any disturbance of metabolism resulting from such treatment. Reducing substances in the urine are not described by the first experimenters, and until recently any workers who found dextrose in the urine after partial asphyxia had experimented on animals breathing in a confined space under such conditions that the partial pressure of oxygen fell and that of carbon dioxide rose simultaneously. It is invariably stated, however, that the glycosuria or hyperglycaemia is due to lack of oxygen, the possible effect of the presence of an increasing amount of carbon dioxide not being taken into consideration apparently.

One of the first to find glycosuria when an animal had been partially asphyxiated was Reynoso (1), while Bernard (2) found that partial asphyxia if prolonged caused a disappearance of the glycogen from the liver, this being accompanied by glycosuria. Dastre (3) found in addition that the sugar of the blood was increased in such cases, the percentage being doubled in the case of a dog after breathing in a confined space for a considerable time. This change was ascribed to lack of oxygen, but obviously towards the end of the experiment the animal would be breathing air containing a considerable percentage of carbon dioxide.

More recently a large number of experiments have been carried out by Araki (4) and by v. Terray (5). Araki seems to be the only one who found dextrose regularly in the urine of animals which had been breathing air containing low percentages of oxygen in absence, as he supposed, of an increased amount of carbon dioxide, while v. Terray kept his animals in a cage which was rapidly ventilated by a mixture of gases containing a low percentage of oxygen and no carbon dioxide. v. Terray only found dextrose in the urine of one animal, and that in very small amount, but his experiments were shorter in duration than Araki's, so that the dextrose in the urine might not have had sufficient time to develop.

Both of these observers were aware of the fact that the work of earlier authors had been largely vitiated by the accumulation of carbon dioxide concurrently with the diminution in oxygen so that these two factors could not be dissociated. They both tried also to avoid the accumulation of carbon dioxide in the apparatus used. It is the more remarkable, therefore that neither Araki nor v. Terray, so far as can be judged from the papers they published, made any analysis of the air breathed by the animals in their experiments. Neither could say definitely, therefore, whether or not an accumulation of carbon dioxide had taken place. That the arrangements for removal of carbon dioxide were not satisfactory is, I think, the reason for the glycosuria obtained by Araki. He caused

the air to circulate continuously through the apparatus, and the carbon dioxide was supposed to be absorbed by a strong solution of potassium hydrate before the air was breathed by the animal in the respiratory chamber again. I have found, however, that this method of absorbing carbon dioxide cannot always be relied on, and as already mentioned Araki gives no analyses of the air breathed.

In v. Terray's experiments the air containing a low percentage of oxygen was sent through the respiratory chamber once only and in this case no great accumulation of carbon dioxide would probably arise. It is to be remembered also that v. Terray only found glycosuria in one animal, the amount of glucose in the urine being at the same time very small.

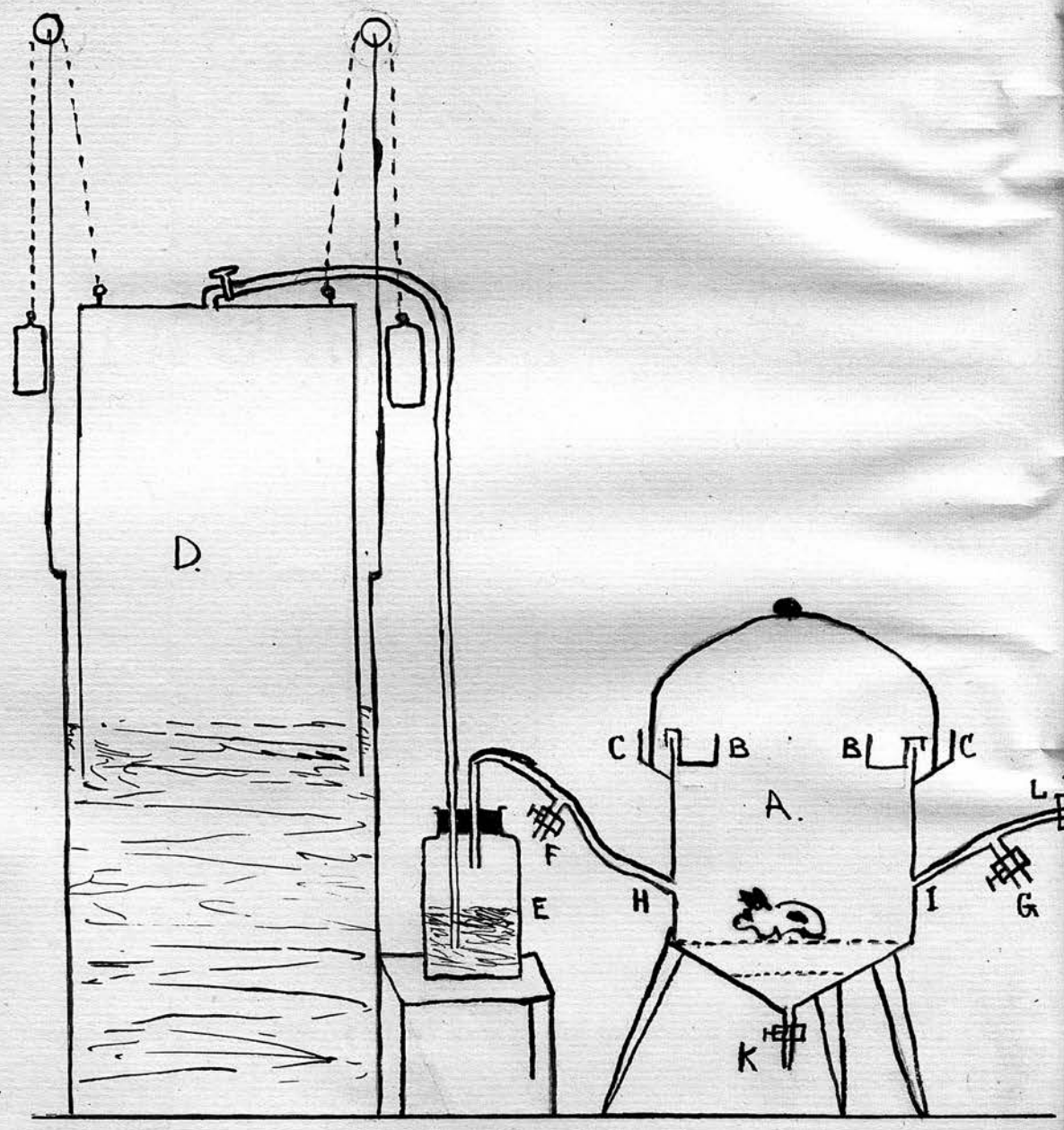
EXPERIMENTAL METHODS.

The apparatus employed in the experiments to be described was devised to maintain a mixture of gases of approximately constant composition throughout an experiment lasting over a day if necessary, without the necessity of a constant circulation of large volumes of air through the respiratory chamber. This implied some way of rapidly removing the carbon dioxide formed by the animal and at the same time keeping the percentage of oxygen practically constant. It was also desired to be able to estimate the amount of oxygen used and of carbon dioxide formed by the animal during the experiment.

The method employed was to place an absorbent for carbon dioxide in the chamber itself, out of reach of the animal, and as the animal used up the oxygen to have the latter gas replaced by pure oxygen drawn in automatically as the pressure in the chamber fell owing to the absorption of the carbon dioxide.

The apparatus was arranged as shown in the accompanying sketch. The respiratory chamber is shown at **A**. It is a cylindrical cage made of tin or galvanised iron, with a conical bottom ending in a narrow tube to which a short piece of rubber tubing is attached. This tubing is closed during an experiment by a screw clip **K**. By opening the clip the urine can be drawn off at any time.

The animal sits on a perforated zinc plate which retains the faeces. Any particles which fall through accidentally are retained by a smaller plate with very fine perforations placed below the plate on which the animal sits.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At B is an annular tray of tin which passes easily down into the cage and is suspended from the upper rim of the cage by three slips of tin attached to it at equal distances apart and turned over at the top so as to hook over the top of the cage. This tray held a quantity of soda-lime in medium sized grains which absorbed the carbon dioxide formed by the animal.

The top of the respiratory chamber consisted of a large bell jar which rested in a circular water seal C in order to make the apparatus air tight.

At H and I are two small metal tubes by which air can be introduced into or extracted from the chamber.

The gasometer D, of about 90 litres capacity, was filled with oxygen (prepared by heating potassium permanganate or by acting on sodium peroxide with dilute sulphuric acid). The amount of oxygen in the gasometer could be determined by the reading on a scale.

When an experiment was in progress the suspension weights of the gasometer were so adjusted that oxygen was just about to bubble through the wash bottle E. Any diminution in the pressure inside the chamber owing to absorption of the carbon dioxide by the soda-lime then caused oxygen to bubble through the wash bottle and enter the chamber at H. When the pressure was again atmospheric the inflow of oxygen ceased for a time until more carbon dioxide had been formed and absorbed.

T pieces at F and G enabled samples of the air in the chamber to be drawn off for analysis. Other gases such as nitrogen could also be introduced thus as will be described later.

When the supply of oxygen in the gasometer was absolutely pure the screw clip L was closed. It usually happened, however, that the gas contained only 95 or 96 per cent of oxygen, the rest being nitrogen. If an animal during a prolonged experiment were allowed to draw over such a mixture to replace the carbon dioxide absorbed, the oxygen content of the chamber would gradually fall and the nitrogen content rise correspondingly. In order to correct this the rubber tubing beyond L was attached to a large aspirator bottle filled with water which was run off gradually. The total air volume was thus gradually increased and the oxygen kept at the desired percentage. Any necessary adjustments were made when an analysis of the air in the chamber showed that the percentage of oxygen was getting too low. With good soda-lime no difficulty was experienced in keeping the percentage of carbon dioxide down to 0·2 or less when desired. When it was desired to keep an animal in an atmosphere free from carbon dioxide and with the percentage of oxygen lower than that of ordinary air, the annular tray was filled with soda-lime and the screw clip at the bottom of the respiratory chamber opened, the end of the rubber tube there being kept under water in a beaker. The air of the chamber was then rapidly flushed out by a current of nitrogen, until analysis of a sample of the air showed the desired percentage of oxygen. The screw clip K was then closed and tne animal allowed to draw in oxygen to replace the carbon dioxide absorbed by the soda-lime.

Frequently at the beginning of an experiment the wash bottle E

was disconnected from D, air instead of oxygen being drawn into the chamber as the carbon dioxide was absorbed. This led to a more gradual reduction of the oxygen percentage than was the case when the apparatus was flushed out with nitrogen. When the desired conditions were reached the wash bottle was again connected with the gasometer and the experiment continued as usual.
When nitrogen was required it was prepared either by heating a mixture of sodium nitrite and ammonium sulphate or by passing air containing ammonia over palladium asbestos contained in a heated combustion tube. The nitrogen was then passed through wash bottles containing (a) hydrochloric acid and (b) sodium hydrate to remove excess of ammonia and nitric acid respectively.
When it was desired to keep an animal in an atmosphere with a high percentage of carbon dioxide no soda-lime was put in the tray and a rapid current of carbon dioxide was led through the chamber along with a current of oxygen until analysis showed that the air in the chamber contained the required proportions of these two gases. The next step was to maintain these proportions as well as possible throughout the whole experiment.
The screw clip L was removed and the rubber rubing there was attached to a water pump. A wash bottle similar to E was then attached to the T piece at F. By now working the water pump a mixture of air and oxygen could be drawn through the chamber, the relative proportions being regulated by screw clips.
By careful regulation and with frequent analyses of the air it was thus possible to arrange that the air breathed by the animal was approximately constant in composition all the time.

DESCRIPTION OF EXPERIMENTS.

Most of the experiments were carried out on rabbits and cats, but dogs and rats were also used. In every case the animal was kept in the chamber for a day or two before the experiment breathing ordinary air, and the urine of this period was examined. In no case was any dextrose found in this urine.

After each experiment also the animals were kept under normal oxygen conditions until the urine was again free from dextrose.

The dextrose was estimated by boiling a measured volume of urine with excess of Fehling's solution and filtering off the precipitated cuprous oxide. This was washed thoroughly and ignited. From the weight of cupric oxide thus obtained the amount of dextrose was found in accordance with the well known table. The presence of dextrose was confirmed in some cases by examination of the osazone obtained from the urine, and in other cases by the fermentation test.

The experiments may be divided into three classes:-

(A) Low percentage of oxygen and high percentage of carbon dioxide in the air breathed.

(B) Low percentage of oxygen and the carbon dioxide kept as low as possible.

(C) Percentage of oxygen as high as in ordinary air, the percentage of carbon dioxide also being high.

(A). Low percentage of oxygen and high percentage of carbon dioxide.

1. A cat of 2·6 kilogrammes was put in the chamber with an amount of soda-lime which happened accidentally to be too small to absorb all the carbon dioxide. The experiment was in connection with an investigation into the effects of varying the percentage of oxygen in the air breathed on the respiratory quotient and on metabolism generally.
The experiment lasted sixteen hours, at the end of which time the air in the chamber contained 7·7 per cent of oxygen and 10·3 per cent of carbon dioxide. During this period 130 c.c. of urine were passed, containing 3·1 per cent of dextrose. A small amount of urine passed four hours afterwards also contained dextrose.
It was the accidental accumulation of carbon dioxide and the subsequent appearance of dextrose in the urine in this experiment that led to the rest of these experiments being carried out.

2. A cat of 2·7 kilogrammes was put in the chamber. No soda-lime was used but 350 c.c. of thirty per cent potassium hydrate were put in the chamber in a shallow flat dish about seven inches in diameter. The cat was allowed to breathe in air to replace the carbon dioxide absorbed until the percentage of oxygen had fallen to about sixteen by the accumulation of nitrogen. At this point the chamber was put in connection with the oxygen reservoir and the experiment continued with

the intention of keeping the animal in air containing a reduced percentage of oxygen but no carbon dioxide. The potassium hydrate, however, failed to absorb the carbon dioxide satisfactorily and after about two hours the air in the chamber contained 10·5 per cent carbon dioxide and only 8·4 per cent oxygen.

Later in the same day the cat was again put in the chamber, a freshly made solution of potassium hydrate being used in the same way as before. In two hours the carbon dioxide had accumulated to the extent of seven per cent and the experiment was stopped. Next morning 120 c.c. of urine were obtained, containing 1·1 per cent of dextrose.

This experiment shows that potassium hydrate cannot be always relied on to absorb carbon dioxide completely, at least in the manner in which I employed it.

(B). Low percentage of oxygen and carbon dioxide also kept as low as possible.

3. A cat of 2·2 kilogrammes was put in the chamber with soda-lime. Nitrogen was sucked in to replace the carbon dioxide absorbed until the percentage of oxygen had fallen to about seven, after which the chamber was connected with the oxygen reservoir. The percentage of oxygen dropped slightly more in the course of the experiment.

Analysis of the air in the chamber during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
2 10 p.m.	6·9	0·2
3 25 "	5·9	0·3
4 30 "	5·0	0·5
5 30 "	5·7	0·6
6 30 "	5·3	0·4

Here the animal was subjected to a lower percentage of oxygen than the cat in Experiment 2, yet, in absence of the high percentage of carbon dioxide it suffered no apparent inconvenience and no glycosuria whatever resulted.

4. A rabbit of 2·7 kilogrammes was put in the chamber with soda-lime. Air was sucked in until the percentage of oxygen fell to about six, when the chamber was connected with the oxygen reservoir.

Analysis of the air in the chamber during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
9 40 a.m.	20·6	0·0
10 45 "	15·3	0·8
1 00 p.m.	6·1	0·9
1 30 "	5·2	0·8
3 00 "	4·2	1·2
4 45 "	5·0	1·1
6 30 "	4·1	0·9
8 30 "	4·3	0·7

In spite of its prolonged exposure to so low a percentage of oxygen the animal appeared to suffer little or no discomfort. At the end of the experiment 92 c.c. of urine were obtained, quite free from dextrose. Next day also the urine contained no dextrose at all.

5. The same rabbit and methods as in the last experiment.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
10 00 a.m.	20·6	0·0
12 noon	11·8	0·9
2 15 p.m.	5·1	0·7
3 45 "	4·3	1·0
6 30 "	4·0	0·5
8 45 "	3·8	0·4
9 15 a.m. (next day)	4·5	0·7

Here the animal after 19 hours exposure to a very low percentage of oxygen showed at the end but little discomfort or dyspnoea. 155 c.c. of urine were passed during this time, quite free from dextrose. The urine obtained afterwards was also normal in this respect.

These two experiments show that in absence of any large amount of carbon dioxide no glycosuria results from prolonged exposure of an animal to air containing the lowest percentage of oxygen compatible with life.

6. A rabbit of 1·7 kilogrammes was put in the chamber with soda-lime. Nitrogen was led in from a reservoir until the percentage of oxygen had fallen below 10. The chamber was then connected with the oxygen reservoir.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
3 30 p.m.	8·8	0·0
8 50 "	7·6	0·1
8 55 a.m.	7·8	0·2
5 00 p.m.	7·6	0·0
8 45 a.m.	7·2	0·7
3 45 p.m.	7·5	0·2

Total duration of experiment 48 hours.

The animal was kept in the chamber during the whole period. Some moist bran was put in at the beginning, of which the rabbit ate at intervals. The urine was drawn off by putting on a little increased pressure inside the chamber and opening the screw clip on the rubber tubing at the bottom of the chamber. In the first 24 hours 75 c.c. of urine were passed, and in the next 24 hours 85 c.c. Both lots were quite free from glucose.

The fact that no trace of glycosuria resulted from keeping an animal for 48 hours in air containing only about a third of the normal percentage of oxygen throws considerable doubt on Araki's results, in view of the fact that in his

papers no analysis of the air breathed is given. Araki's longest experiment lasted about ten hours.

7. A rabbit of 1·7 kilogrammes was put in the chamber with soda-lime. Air was sucked in at first to replace the carbon dioxide absorbed, then the chamber was connected with the oxygen reservoir.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
10 30 a.m.	20·6	0·0
12 30 p.m.	14·5	0·0
4 15 "	8·4	0·0
5 30 "	4·6	0·0
6 30 "	3·0	0·0
6 45 "	4·9	0·0
9 30 "	3·5	0·0

At 6 30 p.m. the animal showed signs of considerable dyspnoea, the percentage of oxygen having dropped to three on account of an accidental constriction on the rubber connection between the chamber and the oxygen reservoir. This was remedied by opening the outflow tube at the bottom of the chamber under water and passing a current of oxygen through the chamber for a short time until the animal recovered.

The rabbit became asphyxiated during the night, the air in the chamber then containing 3·5 per cent of oxygen.

No urine was passed during this experiment, but about 10 c.c.

were obtained by cutting open the bladder. This urine was quite free from dextrose.

8. A rabbit of 1·9 kilogrammes was used, the experiment being carried out in the same way as the last one.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
10 00 a.m.	20·8	0·0
12 15 p.m.	12·7	"
1 30 "	9·7	"
3 15 "	4·1	"
3 45 "	13·3	"
5 30 "	5·4	"
7 00 "	10·0	"
9 30 a.m.	7·3	"

At 3 15 p.m. and at 6 p.m. the oxygen percentage had fallen so low that the rabbit was nearly asphyxiated. At these times, therefore, the chamber was ventilated by a current of oxygen until the animal recovered, the experiment then being continued as before.

Next morning the rabbit was asphyxiated. It had passed about 100 c.c. of urine containing no dextrose. The urine in the bladder at death was also free from dextrose.

9. A rabbit of 1·8 kilogrammes was used, the experiment being carried out in the same way as the last two.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
10 30 a.m.	20·6	0·0
2 15 p.m.	4·8	"
2 45 "	10·0	"
4 15 "	6·6	"
6 30 "	6·3	"
11 00 "	8·3	"

At 2 15 p.m. and again at 6 30 p.m. the rabbit was nearly asphyxiated. A current of oxygen was passed through the chamber on these occasions until the animal recovered.

At the end of the experiment the rabbit was asphyxiated. It had passed 150 c.c. of urine free from dextrose, and the urine in the bladder at death was also free from dextrose.

The last three experiments show that no glycosuria results from keeping rabbits for a considerable time under such conditions that asphyxia afterwards ensues from want of oxygen.

It would also appear that an animal which has been exposed to air so poor in oxygen as to produce partial asphyxia cannot immediately afterwards breathe such a low percentage of oxygen with safety.

10. A cat of 2·7 kilogrammes was put in the chamber with soda-lime and sucked in air to replace the carbon dioxide absorbed until the oxygen fell to about 11 per cent. The chamber was then connected with the oxygen reservoir.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
10 15 a.m.	20·7	0·0
11 15 "	15·2	0·2
12 45 p.m.	9·3	0·3
2 40 "	10·8	0·0
3 50 "	9·7	0·0

The cat suffered considerably from dyspnoea for about three hours and was nearly asphyxiated. About 20 c.c. of urine were obtained at the end of the experiment, quite free from dextrose. The urine next day also was normal.

11. A cat of 2·4 kilogrammes was used as above.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
10 00 a.m.	20·6	0·0
11 00 "	15·6	0·3
12 noon	10·9	0·3
1 45 p.m.	12·4	0·0
3 30 "	11·4	0·0

At 3 30 p.m. the cat was asphyxiated. No urine had been passed during the experiment, but some was obtained by

cutting open the bladder. This urine contained no dextrose. These two experiments show that in the absence of carbon dioxide glycosuria does not result from exposing cats to air so poor in oxygen as to produce asphyxia.

On the whole it was found that rabbits were much more resistant to the effects of low percentages of oxygen than cats. In nearly all the experiments on cats exposure to air containing ten per cent or less of oxygen produced partial asphyxia, whereas rabbits were often hardly affected when the percentage of oxygen in the air breathed fell to five.

(C) Percentage of oxygen as high as in ordinary air, but percentage of carbon dioxide also high.

12. A cat of 2·9 kilogrammes was put in the chamber and a mixture of carbon dioxide and oxygen rapidly aspirated through the apparatus. A mixture of air, oxygen and carbon dioxide was then aspirated through.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
11 45 a.m.	21·3	20·0
12 45 p.m.	21·1	22·7
2 00 "	25·1	18·1
4 15 "	21·2	18·2

The experiment was stopped at 5 p.m. No urine was passed in this period, but next morning at 9 a.m. 225 c.c. of urine were obtained, containing 1·1 per cent of dextrose.

The total amount of dextrose in this urine was 2·48 grammes, which is far in excess of the quantity that could be present as uncombined dextrose in the cat's blood at any one time. The next quantity of urine passed was free from dextrose.

13. A cat of 2·6 kilogrammes was used as above described. Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
12 noon	20·6	0·0
2 45 p.m.	26·7	20·3
4 45 "	24·8	20·5
6 00 "	25·5	18·4

At the end of this period 60 c.c. of urine were obtained, free from dextrose. This had probably been passed soon after the cat was put into the chamber. Next morning another 62 c.c. were obtained, containing 3·64 per cent of dextrose.

These two experiments show that even in the presence of 25 per cent of oxygen glycosuria results in cats if the air breathed contains a high proportion of carbon dioxide.

14. This experiment and tne next are mentioned in order to show the limit of carbon dioxide required to produce glycosuria in cats. This lies between ten and fifteen per cent, varying somewhat with different individuals.

A cat of 2·6 kilogrammes was put in the chamber and a mixture of air and oxygen aspirated slowly through the apparatus.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
2 15 p.m.	20·8	0·0
5 15 "	22·0	5·1
6 15 "	20·2	6·9
8 30 "	15·1	8·2
9 20 "	17·2	7·9

No glycosuria appeared as a result of this experiment. In another experiment with the same cat, in which the carbon dioxide reached 12·4 per cent, 120 c.c. of urine were obtained containing slightly over one per cent of dextrose.

15. A cat of 2·9 kilogrammes was used as in the last experiment.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
12 25 p.m.	9·4	7·9
1 35 "	7·2	9·3
2 45 "	11·2	6·8
4 45 "	9·3	8·4
6 00 "	7·2	9·9

No glycosuria resulted after this experiment.

16. In a second experiment with this cat the carbon dioxide varied between 13 and 15 per cent, and the urine now gave well marked reactions of dextrose.

17. A week later the same cat was used again. The oxygen percentage this time was considerably higher than in the first experiment with this animal. In this case a remarkably pronounced glycosuria resulted.

A mixture of carbon dioxide and oxygen was first led through the apparatus, then a mixture of air and oxygen.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
11 40 a.m.	20·2	14·6
12 5 p.m.	17·6	17·0
1 5 "	17·4	19·5
2 00 "	13·8	19·8
3 20 "	12·5	19·5
4 35 "	10·2	21·9
5 15 "	9·4	21·4

The cat was anaesthetised by the carbon dioxide during the whole of this period.

At the end of this experiment 142 c.c. of urine were obtained, containing no less than 5·42 per cent of dextrose. Next morning another 97 c.c. were obtained, containing 1·03 per cent dextrose, the total amount of dextrose in the urine after this experiment being 8·7 grammes.

18. A rabbit of 2·7 kilogrammes was used, a mixture of oxygen, nitrogen and carbon dioxide being aspirated through the apparatus so as to keep the percentage both of oxygen and carbon dioxide very high.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
10 15 a.m.	20·6	0·0
10 45 "	29·6	9·4
12 00 noon	26·9	14·7
1 35 p.m.	22·5	22·0
1 40 "	21·6	26·5
4 35 "	22·7	26·4
5 30 "	21·4	27·9

No urine was obtained until next morning, when 8 c.c. were passed containing 1·2 per cent of dextrose. On the second morning other 175 c.c. were obtained, containing 0·4 per cent of dextrose.

19. A rabbit of 2·2 kilogrammes was used as above.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
12 00 noon	23·7	11·2
1 00 p.m.	25·1	18·7
2 10 "	22·6	27·4
3 30 "	21·1	24·1
4 45 "	23·1	21·5
5 30 "	21·9	23·1

At the end of this experiment 32 c.c. of urine were passed, containing 2·0 per cent of dextrose. The next urine, 36 hours afterwards, was free from dextrose.

The rabbit was not anaesthetised by that high percentage of carbon dioxide in either of the last two experiments, and no inconvenience of any kind apparently was suffered.

20. A dog of 3·2 kilogrammes was used as described above.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
11 25 a.m.	22·3	19·2
12 15 p.m.	19·0	20·8
1 20 "	22·5	17·7
2 15 "	20·1	20·0

The dog was completely anaesthetised during this period.

At 4 30 p.m. 85 c.c. of urine were obtained, containing 1·81 per cent of dextrose. The next urine was normal.

21. Six white rats were put in the chamber and a mixture of oxygen and carbon dioxide was aspirated through it.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
11 25 a.m.	24·2	9·0
12 00 noon	23·4	20·3
2 15 p.m.	25·3	21·4
3 30 "	27·3	21·1

All the rats were anaesthetised by the carbon dioxide, and

three died during the experiment. Next morning 15 c.c. of urine were obtained from the three surviving rats, and this contained 0·72 per cent of dextrose.

22. Twelve white rats were used as in the last experiment.

Analysis of the air during this experiment:-

Time	Oxygen percentage	Carbon dioxide percentage
12 30 p.m.	29·1	14·9
2 15 "	29·0	18·2
3 45 "	23·3	28·2
4 45 "	23·4	25·6

All the rats were anaesthetised by the carbon dioxide. No urine was obtained that night, but next morning 14 c.c. were obtained, containing 0·84 per cent of dextrose.

DISCUSSION OF RESULTS.

The experiments detailed above show clearly that the glycosuria which frequently accompanies asphyxia is due to excess of carbon dioxide and not to lack of oxygen in the air breathed. Schiff (6) had previously observed that ligature of a limb caused glycosuria and he supposed that the stagnation of the blood in such a case led to the production of an enzyme which increased the formation of dextrose in the liver.

Lepine and Boulud (7) considered Schiff's explanation improbable, and supposed that it was not the stagnation of the blood but the lack of oxygen in the tissues in this case which produced glycosuria by forming "leucomaines" which interfered with the glycolysis of the blood. These observers state, in support of their view, that injection of the blood of a partly asphyxiated animal into a normal animal (guinea-pig) produces glycosuria in the latter, and insist that this effect is due to the "leucomaines" produced by the lack of oxygen.

Ligature of a limb as in these experiments must lead to an accumulation of carbon dioxide in the blood and tissue fluids of the limb and bring about the same conditions locally as occur over the whole animal in asphyxia. The same may be said about the experiments of Lepine and Boulud in which they injected blood from a partially asphyxiated animal, for this blood would contain not only an excess of carbon dioxide, the product of previous asphyxiation, but also an excess of dextrose

set free from the tissues of the asphyxiated animal.

An important point brought out by the experiments described in the present paper is that the amount of dextrose excreted in the urine is far in excess of the total amount of dextrose capable of circulating in the uncombined condition in the animal's blood at any one time. This shows that the agent causing the glycosuria is capable of attacking the dextrose-containing substances present in the tissues and setting free the dextrose from these so as to increase the percentage in the blood and lead to glycosuria.

Thus, in experiment 17, 7·7 grammes of dextrose were excreted in the urine in about five hours. This is twenty or thirty times the amount which would be normally present in the whole of the animal's blood at one time, showing that the excess of carbon dioxide must have led to the setting free of dextrose from some other substance in the tissues.

The view that the glycosuria of asphyxia is caused by excess of carbon dioxide and not by lack of oxygen is supported by the work of Hamburger (8) on the effect of passing carbon dioxide through defibrinated blood. He found that if this were done the percentage of dextrose in the serum after centrifugalising the blood was much higher than in the serum of the same blood which had not been treated with carbon dioxide before being centrifugalised.

Hamburger ascribed the increased amount of dextrose in the serum after treatment with carbon dioxide to the action of

this gas in removing dextrose from the corpuscles and transferring it to the serum.

Experiments carried out by Edie and Spence (9) , however, show that there is practically no dextrose in the corpuscles, and further that the amount of dextrose which can be obtained from the serum by dialysis is increased by treating the serum (after centrifugalising) with carbon dioxide or other anaesthetics. This increase amounted in some cases to thirty per cent of the amount present before treatment with carbon dioxide.

It is well known that dextrose is carried to a considerable extent loosely combined with the serum proteins. The large excretion of dextrose in the urine after administration of phloridzin is due to the liberation of the dextrose from such a compound after an animal has been rendered glycogen-free in various ways.

It is most probable that carbon dioxide produces glycosuria also by breaking down this loose compound of dextrose and protein. In many of these experiments the carbon dioxide was found to act as an anaesthetic, especially in the case of cats and dogs, and it is interesting to note that all anaesthetics which have been tested have been shown to produce glycosuria, such as ether, chloroform, nitrous oxide etc. Experiments on glycosuria after administration of various anaesthetics have been carried out by Edie, Moore and Roaf (10).

It has been shown by Moore and Roaf (11) that anaesthetics

possess the property of adsorbing and combining with the tissue proteins and in this way limiting the power of the protoplasm to combine with other substances for purposes of metabolism. It has also been shown by me (12) that chloroform combines with and in certain cases precipitates haemoglobin and serum protein, the precipitate having a fairly definite proportion of chloroform when the protein is fully saturated therewith.

On this basis a simple explanation is offered for the general action of all anaesthetics in producing glycosuria.

There is little doubt that the carbohydrate of the cells is retained by a process of combination or absorption with the protein of the cells. If now the protein is simultaneously offered any anaesthetic for combination and has a stronger attraction for this, in all probability the carbohydrate previously in combination will be set free and the percentage of dextrose in the blood will be raised and lead to glycosuria.

In the case of carbon dioxide the experiments of Edie and Spence have already been mentioned as showing that carbon dioxide can displace the dextrose from its combination with the serum proteins. It may also be mentioned that Buckmaster (13) found that carbon dioxide is capable of combining loosely with haemoglobin.

SUMMARY.

1. The glycosuria found after partial asphyxia is not due, as hitherto supposed, to lack of oxygen, but to the high percentage of carbon dioxide in the respired air.
2. The presence of from ten to fifteen per cent of carbon dioxide in the respired air leads to glycosuria, and this occurs even if the percentage of oxygen be as high as or higher than that of ordinary air.
3. A low percentage of oxygen alone (less than five) unaccompanied by excess of carbon dioxide never causes glycosuria.
4. The high percentage of carbon dioxide necessary to produce glycosuria causes also complete anaesthesia in cats, dogs and rats. In rabbits glycosuria appears before anaesthesia, but it was not observed whether or not a still higher percentage of carbon dioxide would also produce anaesthesia in this animal.
5. Carbon dioxide falls under the general rule that anaesthetics produce glycosuria.
6. Since it is known that anaesthetics combine with proteins, this suggests that the glucosuria is due to combination taking place between the anaesthetic and the protein, with the consequent liberation of carbohydrate from previous combination with protein. This increase of carbohydrate causes hyperglycaemia and then glycosuria.

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ON THE RESISTANCE OF TRYPSIN SOLUTIONS TO HEAT.

(Originally published in the Bio-Chemical Journal, Vol. 8, No. 1).

The action of heat on aqueous solutions of many enzymes has been studied more or less carefully for many years, and the general conclusion arrived at has been that all enzymes in aqueous solution are destroyed when heated for a short time to about 70° or 75° C. So much is this the case that frequently the activity of a substance after its solution has been heated has been taken as proving that the substance in question is not an enzyme (e.g. secretin).

Since trypsin is practically without any digestive action in acid or neutral solution, the effect of heat on this enzyme was at first tested principally in alkaline solution, and it was found that such a solution rapidly became inactive at as low a temperature as 50° or sometimes even at 45° C. (Biernacki, 1). Vernon (2) also found that fresh preparations of trypsin lost more than half their activity when kept in 0·4 per cent sodium carbonate at 37° C. for an hour. Similar results have been found by Vernon in later experiments, and by other observers. On the other hand Vernon (3) found that the presence of protein protected trypsin solutions from the effect of heat to a considerable extent, and the same protection was afforded by proteoses or peptone. Bayliss and Starling(4) had previously noticed the protective effect of proteins or their hydrolytic products on solutions of trypsin.

The action of acids on trypsin, however, has not been so fully studied. Langley (5) found that trypsin was considerably weakened by warming its solution for 2·5 hours with 0·05 per cent

hydrochloric acid. Wróblewski, Bednarski and Wojczynski (6) found that trypsin when kept at 37° C. for a few hours in hydrochloric acid of over 0·14 per cent was considerably affected, and if 0·56 per cent acid were used the enzyme was sometimes completely destroyed.

The question of the effect of heat on trypsin was investigated later by Schmidt (7) who stated that trypsin in a slightly alkaline solution containing five per cent of peptone could be boiled without being destroyed. The same protection against heat was afforded by a two per cent solution of agar or a ten per cent solution of gelatin. Schmidt also stated that if trypsin powder were suspended in water-free glycerol it could be heated to 292° C. without being destroyed or much affected. Schmidt's work was repeated by de Souza (8) who however found that five per cent peptone had hardly any effect in protecting trypsin solutions from destruction by heat. It appears from these experiments that an appreciable protection is afforded by twenty per cent peptone under certain conditions, but if the heating is sufficient to cause complete destruction of the trypsin in pure aqueous solution, then the presence of peptone has only a slight protective effect. De Souza also tried the effect of heat on trypsin in presence of twenty per cent peptone in acid, neutral and alkaline solutions. The solutions were heated to 80° C. for five minutes. No difference was observed between the acid and neutral solutions, the activity of these after heating being however slightly greater than that of the alkaline solution. Even in the

case of the acid or neutral solution, however, over 85 per cent of the trypsin was destroyed, and about 90 per cent in the case of the alkaline solution. Ohta (9) also repeated the experiments of Schmidt, but failed to confirm his results.

In a paper just published, Mellanby and Woolley (10) find that while trypsin is readily destroyed by heat in neutral or alkaline solution, if a solution of trypsin be made slightly acid, say with hydrochloric acid, it can be boiled for some minutes and yet retain considerable digestive power. According to these observers, trypsin is destroyed in acid more rapidly than in alkaline or neutral solution up to about 40° C. but at higher temperatures the reverse is the case. At 40° C. there appears to be an optimum protective concentration of acid, above or below which the rate of trypsin destruction is accelerated.

Mellanby and Woolley tested the activity of their trypsin solutions by their power to coagulate calcified milk.

Before the publication of the work of Mellanby and Woolley, and unaware of their experiments, I had tested the effect of heat on trypsin in connection with another research, and obtained results of a similar character. The method of testing the digestive power of the trypsin solutions was that of Hedin (11). The trypsin was allowed to act on a solution of caseinogen in the presence of toluene and after a definite interval excess of tannic acid was added, to precipitate unaltered protein, metaprotein and proteoses. After standing at least twelve hours the precipitate was filtered off and the nitrogen determined in a

portion of the filtrate by Kjeldahl's method. Controls were also carried out to show the effect, if any, of the alkali used on caseinogen, and the amount of nitrogen not precipitated by tannic acid was also determined in each solution of trypsin used. The following are the principal results obtained:-

1. Benger's "Liquor Pancreaticus" used as trypsin solution.

   10 cc. of this required 1·6 cc. of N/10 sodium hydrate for neutralisation. 2 per cent caseinogen in fifth normal sodium carbonate was the substrate. A portion of the trypsin was boiled for three minutes and cooled before adding the caseinogen. Digestion was continued at 37° C. for three hours.

	Digestion in cc. of N/10 nitrogen not ppted by tannic acid.
a. 1 cc. trypsin, 20 cc. water, 40 cc. caseinogen	49·8
b. 1 cc. boiled trypsin " "	29·7

   In this experiment the effect of boiling trypsin in slightly acid solution for three minutes was to leave sixty per cent of the original digestive power unimpaired.

2. 10 cc. of the above trypsin solution solution were neutralised with sodium carbonate and made up to 25 cc. with water. Three portions (a, b and c) were boiled for three minutes in neutral, alkaline and acid solution respectively, cooled and kept at 37° C. with 20 cc. of 2 per cent caseinogen in N/5 sodium carbonate for three hours.

	Digestion in cc. of N/10 nitrogen not ppted by tannic acid.
a. 2·5 cc. trypsin, 20 cc. water	0·2
b. 2·5 cc. trypsin, 19 cc. water, 1 cc. N Na_2CO_3	0·1
c. 2·5 cc. trypsin, 19 cc. water, 1 cc. N HCl	20·8
d. 2·5 cc. unboiled trypsin, 20 cc. water	20·9

It may here be mentioned that in all experiments carried out, any differences in reaction due to the trypsin having been boiled in acid etc. were adjusted before the caseinogen was added. Special care was taken also to ensure that none of the trypsin escaped being heated to 100° C.

In the above experiment it will be seen that after being boiled in acid solution for three minutes the trypsin still retained all its power of digesting caseinogen, while boiling in alkaline or neutral solution had completely destroyed the enzyme.

The digestive power of this trypsin before and after being boiled as above was also tested on boiled ox fibrin, the amount of nitrogen in the filtrate from the undissolved fibrin at the end of the digestion being taken as the measure of the action of the enzyme. It was found that on such fibrin trypsin acts only slowly producing much less effect in a given time than when acting on caseinogen. Nevertheless the trypsin boiled in acid dissolved as much fibrin as the unboiled trypsin, while that boiled in neutral or alkaline solution again had no digestive power.

3. Merck's trypsin used. A weak solution of this trypsin was dialysed against running water for 18 hours and filtered. The solution was neutral and contained 0·02 per cent nitrogen. Three portions were boiled for three minutes and then allowed to act on 20 cc. of 2 per cent caseinogen in 0·4 N Na_2CO_3 at 37° C. for three hours.

	Digestion in cc. of N/10 nitrogen.
a. 25 cc. trypsin, 1 cc. N Na_2CO_3	0·0
b. 25 cc. trypsin, 1 cc. water	0·2
c. 25 cc. trypsin, 1 cc. N HCl	21·4
d. 25 cc. unboiled trypsin	28·9

In this experiment 75 per cent of the original digestive power remains after boiling the trypsin in acid solution, but the trypsin is destroyed in neutral or alkaline solution.

For the rest of the experiments the trypsin used was prepared in the manner described by Hedin (12). An ox pancreas was minced and allowed to undergo autolysis at 37° C. in presence of water and toluene for a day and filtered. The filtrate was again kept at 37° for two days, dialysed against running water for two days, filtered and kept with a little toluene. This trypsin solution was neutral, contained less than 0·01 per cent of nitrogen, and gave practically no biuret reaction.

4. Three portions of this trypsin were boiled for three minutes, cooled and allowed to act on 20 cc. of caseinogen as in the last experiment. Digestion lasted three hours.

	Digestion in cc. of N/10 nitrogen.
a. 25 cc. trypsin, 0·5 cc. N Na_2CO_3	0·2
b. 25 cc. trypsin, 0·5 cc. water	0·2
c. 25 cc. trypsin, 0·5 cc. N HCl	6·5
d. 25 cc. unboiled trypsin	10·5

In this experiment we see that over 60 per cent of the original digestive power of the trypsin survives after the acid solution has been boiled, but none in the case of the neutral or alkaline solutions, the 0·2 cc. being within the limits of experimental error.

5. In order to see to what extent this trypsin would survive more prolonged heating, 25 cc. of the solution together with 5 cc. of N/10 HCl were brought to boiling in a flask and then put in a steriliser for 20 minutes. During the whole of this time the temperature throughout the interior of the steriliser was 100° C. The contents of the flask were then cooled and neutralised. To a fresh portion of 25 cc. of the trypsin solution 5 cc. of N/10 HCl were added and immediately neutralised. Then to both flasks were added 20 cc. of the usual caseinogen solution. 25 cc. of water were treated in exactly the same way as the fresh portion of trypsin, and the flasks were kept at 37° C. for 4·25 hours.

	Digestion in cc. of N/10 nitrogen.
a. boiled trypsin	4·2
b. fresh trypsin	16·7
c. control (water)	0·0

The amount of nitrogen not precipitated by tannic acid, contained in the 25 cc. trypsin used, was as usual allowed for in the above results. It corresponded only to 1 cc. of N/10 nitrogen altogether.

From this experiment it appears that under suitable conditions a solution can be heated to 100° C. for fully 20 minutes and still retain 25 per cent of its original digestive power.

6. I have repeated one of the experiments described by Mellanby and Woolley to test the effect of varying concentrations of acid on trypsin. My experiment was carried out at 45° C. at which temperature the acid solutions were kept for 15 minutes. 20 cc. of the usual caseinogen solution were added and digestion continued at 37° C. for three hours.

	Digestion in cc. of N/10 nitrogen.
a. 25 cc. trypsin, 0·2 cc. N HCl	8·0
b. 25 cc. " 0·4 cc. "	8·9
c. 25 cc. " 0·6 cc. "	8·9
d. 25 cc. " 0·8 cc. "	9·3
e. 25 cc. " 1·0 cc. "	8·9
f. 25 cc. unboiled trypsin	9·8

In the above series the trypsin seems to be least protected by the weakest acid (0·008 N), each of the other concentrations of acid having much the same effect. In all cases at least 80 per cent of the original digestive power remains after heating.

SUMMARY.

Solutions of trypsin when neutral or alkaline are rendered completely inactive by boiling.

Acid solutions of trypsin, on the other hand, after being boiled retain a considerable proportion, in some cases the whole of their power to digest caseinogen.

The power to digest caseinogen appears to be less affected by heat than the power to coagulate calcified milk, this being taken as the measure of activity of trypsin by Mellanby and Woolley.

It may be that these two evidences of the action of trypsin are due to different sets of groupings of the trypsin molecule, and that the groupings to which the digestion of caseinogen are due are more thermostable than the others.

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ON THE ACTION OF PEPSIN AND TRYPSIN ON ONE ANOTHER.

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INTRODUCTION.

Hitherto only a small amount of work has been done in investigating the action of enzymes on one another, and many of the earlier results are inconclusive or contradictory. It was stated by Corvisart (1) that pepsin and trypsin were mutually destructive. Kühne (2) on the other hand found that pepsin digested trypsin, but that the reverse did not hold good. A few years later Engesser (3) however, found that pepsin had practically no action on trypsin, digestion being continued for two hours. Further uncertainty was introduced by the work of later authors. Langley (4) tested the action of certain enzymes on one another, taking the precaution to determine the action on acid or alkali of the appropriate strength on the enzymes in question. He found that pepsin was destroyed considerably more rapidly by trypsin in an alkaline solution than by alkali of the same strength acting on the pepsin alone. Langley also found that trypsin lost much of its digestive power when kept for some time in 0·05 per cent hydrochloric acid before being allowed to act on fibrin in alkaline solution, but he does not appear to have done any experiments himself to show the action of pepsin on trypsin.
As the result of a considerable number of experiments on different enzymes, Wróblewski, Bednarski and Wojczynski(5) conclude that not only does trypsin increase the destructive effect of alkali on pepsin, but also that pepsin increases the destructive effect of acid on trypsin. While different observers have found

considerable variations in the range of hydrogen or hydroxyl ion concentration compatible with digestive power on the part of pepsin or trypsin respectively, there seems little doubt that the former enzyme has no appreciable digestive action in an alkaline medium, trypsin on the other hand being inactive in an acid medium. Consequently previous investigations of the action of these enzymes on one another appear to have been confined to discovering if pepsin would digest trypsin in acid solution, or if trypsin on the other hand would have a destructive action on pepsin in alkaline solution.

In the light of the older view that enzymes were of a protein nature it was not unreasonable to expect that in such cases the pepsin for example would in an acid solution break up the trypsin into a number of less complex substances, and that thus the specific digestive properties of the trypsin would be lost. Now that considerable doubt has been thrown on the protein nature of the enzyme molecule, however, such a breakdown of the molecule is by no means certain to take place in every case. Within the last few years the mode of action of enzymes has been rendered clearer by experiments which appear to show that the enzyme acts on its appropriate substrate by means of different chemical groups or side chains. Pollak (6) found that on diluting a trypsin solution with a quantity of the same trypsin solution which had been previously heated to 70° C. much less digestion took place than in the case where the enzyme was diluted with a corresponding amount of water. Similar results

were obtained by Schwartz (7) in the case of pepsin.

Bayliss (8) found that trypsin became altered in the above way on being heated and suggested that the altered enzyme or "zymoid" still retained the power of combining with the substrate, but that the specific digestive power had been destroyed.

Bearn and Cramer (9) also found that pepsin and rennin when heated to about 60° C. inhibited the action of the fresh enzyme when excess of the heated enzyme was added. Generally it was found that if the enzyme were heated to 100° C. this inhibitory power was lost, the reason probably being that the groups which combine with the substrate are themselves destroyed at 100° C. Different enzyme preparations, however, varied considerably in their resistance to heat.

Although trypsin is a more energetic proteolytic enzyme than pepsin, nevertheless they are both capable of acting on a large number of proteins with the formation of similar hydrolytic products up to a certain stage of digestion. This led to the idea that both enzymes might attack proteins by combining with the same side chains, the specific digestive action of the enzymes, however, being due to dissimilar side chains, the latter in the case of pepsin acting only in acid solution, and in the case of trypsin only in presence of alkali. In such a case we should expect excess of trypsin to inhibit the action of pepsin in acid solution, and similarly excess of pepsin would inhibit trypsin in alkaline solution. The enzyme present in excess would thus act in a manner analogous to the zymoid of the other enzyme.

EXPERIMENTAL METHODS.

Various enzyme preparations were used in these experiments, such as the pepsin and trypsin in scales or powder of Merck, Fairchild and Grübler, and extracts of pancreas and stomach prepared in the laboratory by treatment of the finely minced tissues with water containing a little chloroform. Experiments with alcoholic extracts are omitted in this paper, owing to possible complications through the interfering action of the alcohol in some cases. The enzyme solution solutions were put in small flasks and a definite amount of dilute hydrochloric acid or sodium carbonate added. In each flask was then put an equal amount of the protein to be digested and the flasks were kept in a water bath at 37° C. for a certain time, a little toluene or chloroform being added to prevent bacterial action. Any slight accidental variations in temperature would affect all the flasks equally and thus introduce no difficulty.

Two methods of estimating the amount of digestion were employed. The first, principally used for determining the amount of digestion by pepsin, was that of making the mixture of enzymes act on fibrin for a definite time. The undissolved fibrin was then filtered off and the nitrogen in a portion of the filtrate determined by Kjeldahl's method.

Since the object of these experiments was to determine the activity of one enzyme under different conditions, for example the relative digestive power of pepsin in dilute acid and of pepsin in the same strength of acid containing trypsin in addition, such

a method of estimating the amount of digestion seems quite satisfactory, as the stages of digestion must presumably be the same in both cases, and where digestion has gone on for a definite time the amount of dissolved nitrogen must give a true indication of the digestive power of the enzyme under these conditions. The fibrin was always obtained from ox blood, and was minced, thoroughly washed till free from blood, suspended in water and heated to 70° C. in order to destroy any enzymes present. The water was then pressed out and the fibrin preserved in glycerol. When the fibrin was required for use, the glycerol was washed out with water, the excess of water pressed out and equal amounts of fibrin taken for experiment. Control experiments were also carried out with the same amount of fibrin and acid or alkali but no enzyme. No appreciable amount of fibrin was found to be dissolved in any of the controls.

The amount of nitrogen present in the solutions of enzymes used was also determined in every case, and deducted fron the total nitrogen in solution at the end of the experiment. The difference represented the nitrogen of the protein dissolved by the enzyme. The other method of determining digestive power employed in these experiments was that described by Hedin (10). The enzyme is allowed to act on a solution of caseinogen and at the end of the period of digestion excess of tannic acid is added. The unchanged protein, meta-protein and proteoses are precipitated, and the nitrogen in solution is estimated in the filtrate by Kjeldahl's method. This gives a measure of the amount of protein

digested to the stage of peptone or amino-acids.

Control solutions of the enzymes and also of the caseinogen used were also treated in the same way with tannic acid and the nitrogen in the filtrate allowed for in calculating the amount of protein digested. As a rule it was found that the fibrin method was the more suitable when digestion by pepsin was being studied, as pepsin digests caseinogen only very slowly to the stage at which it is not precipitated by tannic acid.

On the other hand the caseinogen method was found more suitable for digestion by trypsin, the latter enzyme dissolving heated ox fibrin much more slowly than does pepsin.

EFFECT OF EXCESS OF TRYPSIN ON THE DIGESTIVE POWER OF PEPSIN.

	Digestion in cc. of N/10 nitrogen.
1. 1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl	10·3
1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	28·6
1·4 g. fibrin added. Digestion 2·5 hours.	
2. 1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl	8·5
1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	31·6
1·5 g. fibrin. Digestion 2 hours.	
3. 1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl	9·2
1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	30.6
1·3 g. fibrin. Digestion 2 hours	
4. 1 cc. pepsin, 10 cc. trypsin, 50 cc. 3N/50 HCl	35·6
1 cc. pepsin, 20 cc. trypsin, 40 cc. 3N/40 HCl	16·2
1 cc. pepsin, 30 cc. trypsin, 30 cc. N/10 HCl	6·1
1 cc. pepsin, 30 cc. water, 30 cc. N/10 HCl	50·8
1·6 g. fibrin, Digestion 2·5 hours.	

	Digestion in cc. of N/10 nitrogen.
5. 1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl	5·2
1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	18·9
1·7 g. fibrin. Digestion 1·25 hours.	
6. 1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl	4·8
1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	13·3
1 g. fibrin. Digestion 2 hours.	
7. 1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl	8·9
1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	22·7
1·1 g. fibrin. Digestion 1·5 hours.	
8. 1 cc. pepsin, 10 cc. trypsin, 50 cc. 3N/50 HCl	7·2
1 cc. pepsin, 20 cc. trypsin, 40 cc. 3N/40 HCl	5·1
1 cc. pepsin, 30 cc. trypsin, 30 cc. N/10 HCl	4·2
1 cc. pepsin, 30 cc. water, 30 cc. N/10 HCl	18·5
1·8 g. fibrin. Digestion 1·5 hours.	

Of these experiments Nos. 1 to 4 were carried out with enzymes prepared in the laboratory, the others being with Merck's pepsin and trypsin (1 per cent solutions).

Exactly similar results were obtained by using Grübler's pepsin and trypsin, and many other experiments with enzymes prepared in the laboratory confirmed in every respect those described above, which, however, are sufficient to show very clearly the effect of an excess of trypsin in inhibiting the digestive action of pepsin solutions. Experiments 4 and 8 also show that this inhibition varies with the proportion of trypsin present. If the amount of the latter enzyme be very large, the action of

the pepsin is almost entirely stopped. This is what was to be expected if we assume that trypsin can combine with protein in acid solution.

The objection might be raised that the action of the trypsin was really due to the presence of protein or the products of protein digestion in the trypsin solutions used. That such was not the case, however, is shown by the following experiments.

	Digestion in cc. of N/10 nitrogen
9. 1 cc. pepsin, 20 cc. 2·5% albumin, 30 cc. N/10 HCl	26·3
1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	25·4
1 g. fibrin. Digestion 1·5 hours.	
10. 1 cc. pepsin. 20 cc. caseinogen, 30 cc. N/30 HCl	29·7
1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	24·9
1 g. fibrin. Digestion 2 hours.	
(The caseinogen was a 2 per cent solution in N/10 HCl).	
11. 1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	31·9
1 cc. pepsin, 20 cc. water, 30 cc. caseinogen	30·7
1 cc. pepsin, 20 cc. water, 30 cc. albumin	37·6
(The caseinogen and albumin were 1·5 per cent solutions in N/10 HCl).	
1·2 g. fibrin. Digestion 1 hour.	
12. 1 cc. pepsin, 20 cc. water, 20 cc. N/10 HCl	25·8
1 cc. pepsin, 20 cc. water, 20 cc. caseinogen	24·4
1 cc. pepsin, 20 cc. water, 20 cc. albumin	26·1
1·1 g. fibrin. Digestion 1·75 hours.	
(The caseinogen and albumin were 1 per cent in N/10 HCl).	

	Digestion in cc. of N/10 nitrogen.
13. 1 cc. pepsin, 20 cc. water, 20 cc. N/10 HCl	32·3
1 cc. pepsin, 20 cc. water, 20 cc. caseinogen	32·6
1 cc. pepsin, 20 cc. water, 20 cc. albumin	31·7

(Caseinogen and albumin were 1 per cent in N/10 HCl).

1·2 g. fibrin. Digestion 0·75 hour.

In experiments 9, 10 and 11, Benger's "Liquor Pepticus" was used. In No. 12, Merck's pepsin, and in No. 13, Grübler's was used. It is clear from these experiments that digestion of a solid protein such as fibrin is not less rapid when there is also present in solution a protein such as caseinogen or egg albumin. The action of the pepsin seems to begin immediately in such a case, there being no interval during which the protein in solution is first broken down into less complex substances. The action of the trypsin is not due to an actual destruction of the pepsin. This is at once seen if the amounts of fibrin vary considerably. It has been shown by Hedin (11) that the amount of caseinogen digested in a given time by a definite amount of trypsin increases with increasing concentration of the substrate, a maximum being reached when sufficient substrate is present to unite with the whole of the trypsin.

I have also obtained similar results in the digestion of fibrin by pepsin.

		Digestion in cc. of N/10 nitrogen
14.	1 cc. pepsin, 20 cc. trypsin, 20 cc. N/10 HCl, 1 g. fibrin	12·3
	1 cc. pepsin, 20 cc. trypsin, 20 cc. N/10 HCl, 3 g. fibrin	35·7
	1 cc. pepsin, 40 cc. N/20 HCl, 1 g. fibrin	28·6
	1 cc. pepsin, 40 cc. N/20 HCl, 3 g. fibrin	71·4
	Digestion 1·5 hours.	
15.	1 cc. pepsin, 20 cc. trypsin, 20 cc. N/10 HCl, 1 g. fibrin	12·7
	1 cc. pepsin, 20 cc. trypsin, 20 cc. N/10 HCl, 4 g. fibrin	45·6
	1 cc. pepsin, 40 cc. N/20 HCl, 1 g. fibrin	30·2
	1 cc. pepsin, 40 cc. N/20 HCl, 4 g. fibrin	89·3
	Digestion 1·5 hours.	
	In these two experiments laboratory preparations were used as enzyme solutions.	
16.	0·8 cc. pepsin, 25 cc. trypsin, 30 cc. N/10 HCl, 1 g. fibrin	7·9
	a. 0·8 cc. pepsin, 25 cc. trypsin, 30 cc. N/10 HCl, 3·5 g. fibrin	17·1
	b. 0·8 cc. pepsin, 25 cc. water, 30 cc. N/10 HCl, 1 g. fibrin	22·4
	0·8 cc. pepsin, 25 cc. water, 30 cc. N/10 HCl, 3·5 g. fibrin	84·5
	Digestion 0·75 hour.	
17.	0·8 cc. pepsin, 25 cc. trypsin, 30 cc. N/10 HCl, 0·7 g fibrin	4·9
	a. 0·8 cc. pepsin, 25 cc. trypsin, 30 cc. N/10 HCl, 5 g. fibrin	12·7
	b. 0·8 cc. pepsin, 25 cc. water, 30 cc. N/10 HCl, 0·7 g. fibrin	18·5
	0·8 cc. pepsin, 25 cc. water, 30 cc. N/10 HCl, 5 g. fibrin	121·9
	Digestion 0·75 hour.	

		Digestion in cc. of N/10 nitrogen
18.	0·8 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 0·5 g. fibrin	4·7
	0·8 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 1·5 g. fibrin	12·6
	0·8 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl, 7 g. fibrin	13·1
	0·8 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl, 0·5 g. fibrin	10·5

Digestion 0·5 hour.

0·5 per cent pepsin and 1 per cent trypsin (Grübler) were used in the last three experiments.

These experiments show well the effect of varying the quantity of fibrin added to the enzyme solutions. It is clear that the effect of adding an excess of trypsin to a given amount of pepsin can be apparently counteracted by increasing the amount of fibrin. The reason evidently is that when the fibrin is increased in amount a larger number of pepsin molecules can carry on their digestive action, although in those experiments at least the amount of digestion by pepsin + trypsin relative to that by pepsin alone is no greater when much fibrin is present than when a smaller amount of the substrate only is used.

In experiments 16 and 17 it will be noticed that the amount of digestion in (a) is still less than that in (b) in spite of the fact that the amount of fibrin in the former is considerably greater than in the latter case. In these two experiments the proportion of trypsin to pepsin is probably higher than in experiments 14 and 15, consequently the proportion of fibrin should have been correspondingly higher in (a) than in (b) to

produce the same effect. By using still smaller amounts of fibrin as in experiment 18 this difficulty is got over, and in this last experiment we see again that by employing suitable proportions of fibrin it is possible to get a greater amount of digestion by the pepsin + trypsin than by the pepsin alone. Since it has been clearly shown that the inhibition of pepsin by excess of trypsin solutions is not due to the presence of soluble protein in the trypsin solution, also that it is not due to the pepsin being destroyed by the trypsin, it seems reasonable to suppose that this inhibition is due to the trypsin itself. If this were the case, we should expect that such an inhibition would no longer occur if the trypsin were destroyed. The simplest way appeared to be by boiling the solution of trypsin before adding it to the pepsin. This, however, proved somewhat ineffective as the following results show.

	Digestion in cc. of N/10 nitrogen
19. 1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl	8·3
1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl (boiled)	15·7
1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	27·2
1·5 g. fibrin. Digestion 2 hours.	
20. 1 cc. pepsin, 20 cc. trypsin, 30 cc. N/10 HCl	12·6
1 cc. pepsin, 20 cc. trypsin 30 cc. N/10 HCl (boiled)	18·3
1 cc. pepsin, 20 cc. water, 30 cc. N/10 HCl	38·7
1·5 g. fibrin. Digestion 2 hours.	

Pepsin and trypsin prepared in the laboratory from sheep's tissues were used in these two experiments. The trypsin was boiled under a reflux condenser for half an hour and filtered.

Trypsin solutions frequently develop a slight acidity on being kept for a short time, and as is now known to be the case, in acid solution this enzyme resists destruction by heat for a considerable time. In the last two experiments, therefore, the trypsin was probably not entirely destroyed, at least as far as its power of combining with fibrin was concerned.

I have found that even in neutral solution a solution of trypsin can be boiled for an hour and will still inhibit the action of the same trypsin unboiled, showing that the zymoid of trypsin is much more resistant to heat than is the zymoid of pepsin, according to Bearn and Cramer. These observers found that different preparations of pepsin varied considerably in their resistance to heat, and different trypsin solutions also vary considerably.

Trypsin was also found to inhibit the action of pepsin on caseinogen solutions, but the action of pepsin on this substrate is very slight, so far at least as rendering the nitrogen non-precipitable by tannic goes.

	Digestion in cc. of N/10 nitrogen
21. 1 cc. pepsin, 20 cc. trypsin, 20 cc. N/10 HCl	2.7
1 cc. pepsin, 20 cc. water, 20 cc. N/10 HCl	6·4

20 cc. 1 per cent caseinogen in N/20 HCl added.

Digestion 3 hours.

This experiment shows that trypsin again inhibits pepsin, but caseinogen is digested too slowly to be a satisfactory substrate for digestion by pepsin.

EFFECT OF EXCESS OF PEPSIN ON THE DIGESTIVE POWER OF TRYPSIN.

The experiments were carried out similarly to those already described. Pepsin and trypsin (sheep) prepared in the laboratory were used throughout.

	Digestion in cc. of N/10 nitrogen
22. 1 cc. trypsin, 20 cc. pepsin, 40 cc. 1% Na_2CO_3	8·5
1 cc. trypsin, 20 cc. water, 40 cc. 1% Na_2CO_3	19·7
1 g. fibrin. Digestion 2 hours.	
23. 1 cc. trypsin, 20 cc. pepsin, 40 cc. 1% Na_2CO_3	11·3
1 cc. trypsin, 20 cc. water, 40 cc. 1% Na_2CO_3	27·8
1·5 g. fibrin. Digestion 2·5 hours.	
24. 1 cc. trypsin, 10 cc. pepsin, 20 cc. water, 35 cc. 1% Na_2CO_3	21·8
1 cc. trypsin, 20 cc. pepsin, 10 cc. water, 35 cc. 1% Na_2CO_3	17·6
1 cc. trypsin, 30 cc. pepsin, 35 cc. 1% Na_2CO_3	10·2
1 cc. trypsin, 30 cc. water, 35 cc. 1% Na_2CO_3	26·3
1·3 g. fibrin. Digestion 2 hours.	

It will be seen from the above that excess of pepsin inhibits the action of trypsin on fibrin, the amount of inhibition varying with the amount of pepsin added.

It was generally found that much more digestion was effected by trypsin when caseinogen was the substrate, the nitrogen being estimated in the filtrate from the precipitate produced by adding excess of tannic acid.

	Digestion in cc. of N/10 nitrogen
25. 1 cc. trypsin, 20 cc. pepsin, 40 cc. caseinogen	20·4
1 cc. trypsin, 20 cc. water, 40 cc. caseinogen	53·7

Caseinogen was 2 per cent in 1% Na_2CO_3

Digestion 2 hours.

26. 1 cc. trypsin, 20 cc. pepsin, 40 cc. caseinogen	12·2
1 cc. trypsin, 20 cc. water, 40 cc. caseinogen	40·6

Caseinogen as above. Digestion 1·5 hours.

The inhibition by the pepsin is well seen in these experiments. The effect of a soluble protein such as caseinogen on the digestion of fibrin by trypsin was also tested.

	Digestion in cc. of N/10 nitrogen
27. 1 cc. trypsin, 20 cc. caseinogen, 20 cc. N/10 Na_2CO_3	30·7
1 cc. trypsin, 40 cc. N/10 Na_2CO_3	28·5

1 g. fibrin. Digestion 2 hours.

Caseinogen was 2 per cent in N/10 Na_2CO_3

28. 1 cc. trypsin, 20 cc. caseinogen, 20 cc. N/10 Na_2CO_3	25·4
1 cc. trypsin, 40 cc. N/10 Na_2CO_3	25·6

1 g. fibrin. Digestion 2 hours.

Caseinogen as above.

These two experiments show that the presence of excess of a soluble protein such as caseinogen does not delay the digestion of fibrin by trypsin. Egg albumin, as is well known, inhibits the action of trypsin, and I found that only a small amount of fibrin was digested if albumin was added in place of the caseinogen in these two experiments. The above results, however, show

that the inhibition of trypsin by excess of pepsin is not due to the trypsin digesting the proteins in solution before attacking the fibrin.

Several experiments were carried out to see if pepsin solutions which had been boiled lost their power of inhibiting the action of trypsin. The results varied somewhat, but generally the inhibitory power of pepsin was much reduced by boiling.

	Digestion in cc. of N/10 nitrogen
29. 1 cc. trypsin, 20 cc. pepsin, 40 cc. caseinogen	16·2
1 cc. trypsin, 20 cc. pepsin (boiled) 40 cc. caseinogen	43·6
1 cc. trypsin, 20 cc. water, 40 cc. caseinogen	43·7

Caseinogen was 2 per cent in N/10 Na_2CO_3

Digestion 1·5 hours.

In some cases the inhibitory power of the pepsin was considerably less completely destroyed by boiling (in the above case for over half an hour), but as has been already mentioned pepsin has been found by other observers to vary considerably in its behaviour to heat, and in some cases the power of combining with protein is more difficult to destroy than in others.

It may be mentioned also that Bayliss found that he could not always produce the zymoid modification of trypsin, or at least demonstrate the action of the zymoid, as though in his experiments considerable differences in the enzymes were met with.

SUMMARY.

Excess of trypsin inhibits the digestive action of pepsin in acid solution. This is not due simply to the presence of protein associated with the trypsin, nor to the pepsin being actually destroyed by the trypsin.

The power to inhibit pepsin is to some extent lost if the trypsin is boiled first, but the zymoid modification of trypsin is in some cases very resistant to destruction by heat.

It is suggested that trypsin can combine with protein in acid solution, so that although unable to digest that protein, an excess of trypsin would prevent pepsin from combining fully with the protein.

Similarly an excess of pepsin inhibits the action of trypsin in alkaline solution. This inhibition again is not simply due to the presence of protein associated with the pepsin. Heating the pepsin destroys in some cases the whole of its power to inhibit the action of trypsin.

In both cases the amount of inhibition produced depends on the relative proportions of the enzymes present. Thus, the greater the amount of pepsin present, the less the digestion which will be effected by a given amount of trypsin in alkaline solution.

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ON THE EFFECT OF ALCOHOL ON THE DIGESTION OF FIBRIN AND CASEINOGEN BY TRYPSIN.

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INTRODUCTION.

The behaviour of extracts of pancreas or pure pancreatic juice under different conditions has led various observers to conclude that the pancreas contains a number of proteolytic enzymes. It was shown by Fermi (1) that after treatment with mercuric chloride, salicylic acid and various other substances, trypsin lost its power of digesting fibrin but would still digest gelatin. Vernon (2) arguing from the varying sensitiveness of pancreatic extracts towards sodium carbonate, concluded that "trypsin" was really a mixture of enzymes of different degrees of stability, the more sensitive enzymes being destroyed first. Vernon only tested the digestive power of trypsin on raw fibrin, however. In a later paper Vernon (3) states that pancreatic extracts contain an erepsin as well as trypsin. Pollak (4) using different preparations of enzymes found that the relative amounts of serum and gelatin digested varied enormously in different cases. He also found that after treatment with hydrochloric acid trypsin lost its power of acting on serum, but was still about as active as ever on gelatin. Pollak concluded that extracts of pancreas contained in addition to trypsin (to which the action on serum was due) a special enzyme which acted only on gelatin. To this enzyme Pollak gave the name of "glutinase".
According to Ascoli and Neppi (5) however, this assumption of a special enzyme acting on gelatin is unjustified, as they find that slight variations in the reaction of the medium affect the

digestion of different proteins to different degrees. Mays (6) after a long series of experiments remarked that the presence of two proteolytic enzymes in pancreatic extracts can only be proved when it is possible to make a separation of the enzymes. It had been previously shown by Bayliss and Starling (7) that pancreatic juice as secreted contains no trypsin (as tested on coagulated egg white) but contains a weak enzyme like erepsin. This has some action on caseinogen, but very slight.

The erepsin has a slight action on fresh fibrin but practically none on fibrin which has been heated to 70° C. It may here be mentioned that Long and Barton (8) state that raw fibrin even when very carefully purified may soon become liquid owing to autolysis and hence is unsuitable as a substrate in experiments on proteolytic enzymes.

In later papers Fermi (9, 10) contests the theory that some proteolytic enzymes have a specific action, and maintains that all such enzymes have a general action on all proteins.

Slight differences of behaviour of trypsin towards different proteins under the same conditions have also been noted by Berg and Gies (11), Porter (12), Long and Hull (13), but not much importance seems to have been attached to the facts.

Others such as Glaessner and Stauber (14) and Auerbach and Pick (15) find differences between the proteolytic and peptolytic actions of trypsin, but in these cases possibly some of the action was due to pancreatic erepsin also.

It seems to have been assumed, however, by all the authors quoted and by others such as Hedin (16) that trypsin is the enzyme responsible for the digestion of fibrin and caseinogen, especially in experiments lasting only a few hours.

The action of alcohol on trypsin has been variously stated. Fermi and Pernossi (17) using Mett's tubes filled with gelatin found that in presence of alcohol trypsin had more digestive action than in presence of water only. The percentage of alcohol used in their experiments is not stated. Chittenden and Mendel (18) found that the action of trypsin on fibrin was markedly inhibited by alcohol, but did not test the action on any other substrate. Dastre (19) found that trypsin still digested fibrin and boiled albumin in presence of fifteen to twenty per cent of alcohol, while Gizelt (20, 21) states that twenty per cent alcohol totally inhibits trypsin. According to Bayliss (22) trypsin will digest gliadin even in presence of eighty per cent alcohol, the action in this case being due to the trypsin in suspension. Vernon (23) noted that dilute alcohol had a considerable inhibitory effect on the digestion of raw fibrin by trypsin. As dilute alcohol is frequently used in making extracts of various digestive organs, it is important to know how the digestive action is affected thereby.

EXPERIMENTAL DETAILS.

The experiments were carried out as described previously (24). Ox fibrin after being finely minced and thoroughly washed was suspended in water and heated gradually to 85° C. The fibrin was then pressed dry and preserved in glycerol and a little chloroform until required. The caseinogen was a three per cent solution in one per cent sodium carbonate. The pancreatic extracts were prepared by finely mincing sheep's pancreas and extracting with water containing a little chloroform for about a fortnight. The extracts were then filtered and a little chloroform added as a preservative.

The digestion was carried on at 37° C. in small flasks, a small measured quantity of chloroform being added to exclude bacterial action in every case. When fibrin was used, the amount of digestion was estimated by filtering off the undissolved fibrin and determining the nitrogen in the filtrate by Kjeldahl's method. When caseinogen was the substrate, the amount of digestion was found by precipitation with tannic acid and subsequent estimation of the nitrogen in the filtrate. Controls showed that the sodium carbonate alone had no digestive action whatever either on fibrin or on caseinogen.

The following are typical results showing the effect of dilute alcohol on the digestion of fibrin and caseinogen by trypsin.

	Digestion in cc. of N/10 nitrogen.
1. a. 1 cc. trypsin, 20 cc. 12% alcohol, 20 cc. 1% Na_2CO_3	4·6
b. 1 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	13·8
1 g. fibrin added. Digestion 2·75 hours.	
c. 1 cc. trypsin, 20 cc. 12% alcohol, 20 cc. caseinogen	24·1
d. 1 cc. trypsin, 20 cc. water, 20 cc. caseinogen	24·3
Digestion 1·25 hours.	
2. a. 1 cc. trypsin, 20 cc. 10% alcohol, 20 cc. 10 Na_2CO_3	4·6
b. 1 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	17·1
1·4 g. fibrin. Digestion 3 hours.	
c. 1 cc. trypsin, 20 cc. 10% alcohol, 20 cc. caseinogen	23·8
d. 1 cc. trypsin, 20 cc. water, 20 cc. caseinogen	23·6
Digestion 1 hour.	
3. a. 1 cc. trypsin, 20 cc. 10% alcohol, 20 cc. 1% Na_2CO_3	3·8
b. 1 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	14·1
1·3 g. fibrin. Digestion 2 hours.	
c. 1 cc. trypsin, 20 cc. 10% alcohol, 20 cc. caseinogen	27·9
d. 1 cc. trypsin, 20 cc. water, 20 cc. caseinogen	27·0
Digestion 1·25 hours.	
4. a. 1 cc. trypsin, 20 cc. 10% alcohol, 20 cc. 10 Na_2CO_3	8·1
b. 1 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	23·6
1·5 g. fibrin. Digestion 2·25 hours.	
c. 1 cc. trypsin, 20 cc. 10% alcohol, 20 cc. caseinogen	30·0
d. 1 cc. trypsin, 20 cc. water, 20 cc. caseinogen	29·3
Digestion 1 hour.	

	Digestion in cc. of N/10 nitrogen.
5. a. 1 cc. trypsin, 20 cc. 8% alcohol, 20 cc. 1% Na_2CO_3	5·1
b. 1 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	12·1
1 g. fibrin. Digestion 3 hours.	
c. 1 cc. trypsin, 20 cc. 8% alcohol, 20 cc. caseinogen	30·6
d. 1 cc. trypsin, 20 cc. water, 20 cc. caseinogen	30·6
Digestion 1·25 hours.	
6. a. 1 cc. trypsin, 20 cc. 6% alcohol, 20 cc. 1% Na_2CO_3	4·9
b. 1 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	12·1
1 g. fibrin. Digestion 3 hours.	
c. 1 cc. trypsin, 20 cc. 6% alcohol, 20 cc. caseinogen	27·7
d. 1 cc. trypsin, 20 cc. water, 20 cc. caseinogen	28·0
Digestion 1 hour.	
7. a. 1 cc. trypsin, 20 cc. 6% alcohol, 20 cc. 1% Na_2CO_3	11·5
b. 1 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	20·3
1 g. fibrin. Digestion 3 hours.	
c. 1 cc. trypsin, 20 cc. 6% alcohol, 20 cc. caseinogen	22·2
d. 1 cc. trypsin, 20 cc. water, 20 cc. caseinogen	22·0
Digestion 1 hour.	
8. a. 1 cc. trypsin, 20 cc. 6% alcohol, 20 cc. 1% Na_2CO_3	12·5
b. 1 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	22·2
1 g. fibrin. Digestion 1 hour.	
c. 1 cc. trypsin, 20 cc. 6% alcohol, 20 cc. caseinogen	25·0
d. 1 cc. trypsin, 20 cc. water, 20 cc. caseinogen	24·6
Digestion 1 hour.	

	Digestion in cc. of N/10 nitrogen.
9. a. 5 cc. trypsin, 20 cc. 16% alcohol, 20 cc. 1% Na_2CO_3	7·1
b. 5 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	18·2
1 g. fibrin. Digestion 2 hours.	
c. 5 cc. trypsin, 20 cc. 16% alcohol, 20 cc. caseinogen	31·6
d. 5 cc. trypsin, 20 cc. water, 20 cc. caseinogen	31·9
Digestion 1 hour.	
10. a. 5 cc. trypsin, 20 cc. 14% alcohol, 20 cc. 1% Na_2CO_3	13·7
b. 5 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	26·8
1·2 g. fibrin. Digestion 2·5 hours.	
c. 5 cc. trypsin, 20 cc. 14% alcohol, 20 cc. caseinogen	32·5
d. 5 cc. trypsin, 20 cc. water, 20 cc. caseinogen	32·5
Digestion 1 hour.	
11. a. 5 cc. trypsin, 20 cc. 14% alcohol, 20 cc. 1% Na_2CO_3	10·8
b. 5 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	24·6
1 g. fibrin. Digestion 2 hours.	
c. 5 cc. trypsin, 20 cc. 14% alcohol, 20 cc. caseinogen	30·0
d. 5 cc. trypsin, 20 cc. water, 20 cc. caseinogen	30·1
Digestion 1 hour.	
12. a. 5 cc. trypsin, 20 cc. 13% alcohol, 20 cc. 1% Na_2CO_3	14·0
b. 5 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	25·2
1·3 g. fibrin. Digestion 3 hours.	
c. 5 cc. trypsin, 20 cc. 13% alcohol, 20 cc. caseinogen	34·5
d. 5 cc. trypsin, 20 cc. water, 20 cc. caseinogen	34·6
Digestion 1·25 hours.	

These experiments are sufficient to show that alcohol, when present in percentages varying from three to seven, has a very marked inhibitory effect on the digestion of fibrin by trypsin but no such effect on the digestion of caseinogen. The amount of fibrin digested under these conditions varied from about 25 to 50 per cent of the amount digested in absence of alcohol, the proportion varying somewhat with different trypsin solutions and with varying percentages of alcohol. In no case was there any appreciable difference in the amount of caseinogen digested, beyond the limits of experimental error.

With higher percentages of alcohol the digestion of fibrin was in some cases entirely stopped, a fair amount of caseinogen still being digested, however.

	Digestion in cc. of N/10 nitrogen.
13. a. 1 cc. trypsin, 20 cc. 25% alcohol, 20 cc. 1% Na_2CO_3	1·3
b. 1 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	27·2
1 g. fibrin. Digestion 3 hours.	
c. 1 cc. trypsin, 20 cc. 25% alcohol, 20 cc. caseinogen	20·0
d. 1 cc. trypsin, 20 cc. water, 20 cc. caseinogen	23·7
Digestion 1 hour.	
14. a. 1 cc. trypsin, 20 cc. 25% alcohol, 20 cc. 1% Na_2CO_3	4·8
b. 1 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	25·2
0·8 g. fibrin. Digestion 3 hours.	
c. 1 cc. trypsin, 20 cc. 25% alcohol, 20 cc. caseinogen	32·8
d. 1 cc. trypsin, 20 cc. water, 20 cc. caseinogen	37·9
Digestion 1·75 hours.	

		Digestion in cc. of N/10 nitrogen.
15.	a. 1 cc. trypsin, 20 cc. 50% alcohol, 20 cc. 1% Na_2CO_3	0·0
	b. 1 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	23·6
	1 g. fibrin. Digestion 3 hours.	
	c. 1 cc. trypsin, 20 cc. 50% alcohol, 20 cc. caseinogen	5·3
	d. 1 cc. trypsin, 20 cc. water, 20 cc. caseinogen	25·2
	Digestion 1 hour.	
16.	a. 1 cc. trypsin, 20 cc. 50% alcohol, 20 cc. 1% Na_2CO_3	0·0
	b. 1 cc. trypsin, 20 cc. water, 20 cc. 1% Na_2CO_3	23·4
	1·2 g. fibrin. Digestion 3 hours.	
	c. 1 cc. trypsin, 20 cc. 50% alcohol, 20 cc. caseinogen	4·6
	d. 1 cc. trypsin, 20 cc. water, 20 cc. caseinogen	28·2
	Digestion 1 hour.	

These experiments show that in presence of 25 per cent alcohol the digestion of fibrin by trypsin is entirely inhibited, while digestion of caseinogen still proceeds to a limited extent.

In presence of 12 per cent alcohol the amount of fibrin digested is from 10 to 20 per cent of the control, while the caseinogen digested amounts to about 85 per cent of the control.

Trypsin is well known to be very unstable under some circumstances, and it was considered possible that contact with dilute alcohol for some time might lead to an actual destruction of the part of the enzyme molecule which digests fibrin. The following experiments were carried out to test such a theory.

	Digestion in cc. of N/10 nitrogen.
17. a. 20 cc. trypsin, 15 cc. 15% alcohol } kept at 37° C. for 3 hours.	
b. 20 cc. trypsin, 15 cc. water }	
2 cc. of (a), 40 cc. 0·5% Na_2CO_3	31·2
2 cc. of (b), 40 cc. 0·5% Na_2CO_3	31·0
1·3 g. fibrin. Digestion 3 hours.	
18. a. 20 cc. trypsin, 5 cc. 30% alcohol } kept at 37° C. for 3 hours.	
b. 20 cc. trypsin, 5 cc. water }	
2 cc. of (a), 40 cc. 0·5% Na_2CO_3	18·2
2 cc. of (b), 40 cc. 0·5% Na_2CO_3	18·3
1·2 g. fibrin. Digestion 2 hours.	
19. a. 40 cc. trypsin, 10 cc. 30% alcohol } kept at 37° C. for 3 hours.	
b. 40 cc. trypsin, 10 cc. water }	
1 cc. of (a), 40 cc. 0·5% Na_2CO_3	14·0
1 cc. of (b), 40 cc. 0·5% Na_2CO_3	14·2
1 g. fibrin. Digestion 3 hours.	
20. a. 15 cc. trypsin, 10 cc. 15% alcohol } kept at 37° C. for 3 hours.	
b. 15 cc. trypsin, 10 cc. water }	
2 cc. of (a), 40 cc. 0·5% Na_2CO_3	17·4
2 cc. of (b), 40 cc. 0·5% Na_2CO_3	17·0
1 g. fibrin. Digestion 2·75 hours.	
21. a. 15 cc. trypsin, 10 cc. 15% alcohol } kept at 37° C. for 3 hours.	
b. 15 cc. trypsin, 10 cc. water }	
2 cc. of (a), 40 cc. 0·5% Na_2CO_3	16·3
2 cc. of (b), 40 cc. 0·5% Na_2CO_3	16·2
1 g. fibrin. Digestion 2·75 hours.	

No destruction whatever of the trypsin is caused by the action of six per cent alcohol, although the digestive action of the enzyme (on fibrin) is reduced to thirty per cent or less of the normal amount by the presence of this proportion of alcohol. A solid substrate such as fibrin might be rendered less digestible by prolonged treatment with concentrated alcohol, owing to the hardening thus brought about. Alcohol of under thirty per cent, however, could hardly be supposed to have such an effect, and a few experiments showed that after treatment with dilute alcohol fibrin was no less digestible by trypsin than previously.

	Digestion in cc. of N/10 nitrogen.
22. a. Fibrin + 10% alcohol } kept at 37° C. for 3 hours.	
b. Fibrin + water }	
1 cc. trypsin, 40 cc. 0·5% Na_2CO_3, 1 g. fibrin (a)	19·3
1 cc. trypsin, 40 cc. 0·5% Na_2CO_3, 1 g. fibrin (b)	18·1
Digestion 2·5 hours.	
23. a. Fibrin + 10% alcohol } kept at 37° C. for 19 hours.	
b. Fibrin + water }	
1 cc. trypsin, 40 cc. 0·5% Na_2CO_3, 1 g. fibrin (a)	22·9
1 cc. trypsin, 40 cc. 0·5% Na_2CO_3, 1 g. fibrin (b)	20·2
Digestion 3 hours.	

The fibrin which was to be treated with alcohol in these experiments was first washed with alcohol so as to remove any adherent moisture. It will be seen that after treatment with ten per cent alcohol fibrin is no less readily attacked by trypsin than

previously, but indeed slightly more readily.

The action of trypsin on fibrin and on caseinogen is affected by dilute alcohol to such different degrees that it is reasonable to suppose either that there are two enzymes concerned in the digestion of these proteins or that different groups of the same enzyme molecule take part in the hydrolysis of the different proteins. In the latter case the groups which digest fibrin are very much more easily inhibited by alcohol than the groups which digest caseinogen.

The theory that different side chains in the molecule of an enzyme are responsible for different functions is used to explain the zymoid modification of enzymes. Some observers also, for example Nencki and Sieber (25) hold that the behaviour of pepsin and rennin under varying conditions can best be explained on the theory that only one enzyme is concerned here, with different side chains responsible for the proteolytic and milk coagulating functions. Vernon (23) also considers this probable in the case of the proteolytic and milk coagulating functions of trypsin.

Hitherto it has apparently been assumed that one enzyme"trypsin" is responsible for the digestion both of fibrin and of caseinogen by pancreatic extracts. In this case the function is the same (hydrolysis of a protein to form simpler products), but it would seem that different side chains may be necessary for the hydrolysis of different proteins.

SUMMARY.

Alcohol when present to the extent of three per cent and over markedly inhibits the action of trypsin on fibrin. The digestion of caseinogen by trypsin is not affected to any extent until the concentration of alcohol reaches ten per cent.

The action of alcohol is not due to the destruction of the power of trypsin to digest fibrin, since on suitable dilution of a mixture of trypsin and alcohol the digestion of fibrin is as great as in the control.

Fibrin is not rendered less digestible by contact with dilute alcohol, but rather slightly more digestible.

If "trypsin" is a single enzyme the digestion of fibrin and of caseinogen are probably carried on by different side chains, those digesting fibrin being much more readily affected by alcohol than those which digest caseinogen.

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FURTHER OBSERVATIONS ON THE DIGESTION OF FIBRIN AND CASEINOGEN BY TRYPSIN, AND ON THE RELATION OF TRYPSIN TO PANCREATIC RENNIN.

In a previous paper (1) it was shown that the activity of trypsin as measured by its action on fibrin and on caseinogen is affected to such a different extent by alcohol as to make it seem either that two enzymes are concerned here or, if only one enzyme, then the two substrates are acted on by different groups or side chains one set of which is very much more sensitive than the other.

The effect of heat on the digestion of fibrin and caseinogen by trypsin was next studied. It had been proved previously (2) that trypsin when boiled in acid solution still retained much or in some cases all of its power of hydrolysing caseinogen, but in only one case was its action on fibrin tested after being heated in this way. Digestion of the fibrin was still as great as in the control with unboiled trypsin, but the amount digested in this experiment was so small that a fresh series of experiments was undertaken. The trypsin solutions were prepared in the laboratory, generally by extracting minced sheep's pancreas with water containing chloroform for ten days or a fortnight and filtering. A little chloroform was then added as a preservative. The experiments were carried out as described in the previous paper. It had been found in the earlier series that in presence of N/25 to N/50 hydrochloric acid the trypsin solutions employed retained from 60 to 100 per cent of their digestive power on caseinogen after being heated to 100° C. for three minutes. The same treatment was applied to the trypsin solutions in this series, with the following results.

In each case 1 cc. trypsin + 40 cc. 0·5% Na_2CO_3 + 1 g. fibrin was used on the one hand, digestion being for 3 hours, and on the other hand 1 cc. trypsin + 40 cc. 1·5% caseinogen in 0·5% Na_2CO_3, digestion being for 1 hour. The amount of digestion in each case was estimated as described in the previous papers of this series.

No.	Reaction (HCl)	Treatment	Fibrin digested in cc. of N/10 nitrogen	Caseinogen digested
1.	N/55	3 minutes at 100° C.	0·0	1·2
		Control	20·1	28·6
2.	N/20	1·5 minutes at 60° C.	0·0	3·1
		Control	21·9	34·7
3.	N/20	1·5 minutes at 60° C.	0·0	5·1
		Control	22·1	30·9
4.	N/30	1 minute at 85° C.	0·0	1·9
		Control	7·1	22·4
5.	N/30	1 minute at 65° C.	0·0	3·2
		Control	8·6	22·9
6.	N/20	0·75 minutes at 100° C.	0·0	3·2
		Control	21·2	34·6
7.	N/20	0·75 minutes at 66° C.	2·2	19·5
		Control	16·6	34·3
8.	N/20	1·5 minutes at 66° C.	1·1	8·3
		Control	16·2	31·8
9.	N/40	1·5 minutes at 75° C.	3·4	17·0
		Control	17·4	37·4

In the above experiments it will be seen that even in acid

solution the pancreatic extracts used generally lost practically all their power to digest fibrin when heated to 60° C. or over for a short time. The power to digest caseinogen was not so completely destroyed, especially in the last three experiments, but was markedly reduced, compared with what had been found previously. The reason for so great a difference was investigated in several ways. The extracts used above were aqueous, so a few experiments were carried out to compare the effect of heat on aqueous and alcoholic (20 per cent) extracts of the same pancreas.

No.	Reaction (HCl)	Extract	Treatment	Fibrin digested	Caseinogen digested
10.	N/40	Aqueous	1·5 min. at 75° C.	3·4	17·0
		"	Control	17·4	37·4
		Alcohol	1·5 min. at 75° C.	17·3	29·3
		"	Control	31·0	47·2
11.	N/50	Alcohol	1 min. at 100° C.	23·4	34·4
		"	Control	27·9	42·8
12.	N/40	Aqueous	1 min. at 100° C.	0·0	
		"	Control	11·8	
		Alcohol	1 min. at 100° C.	21·1	
		"	Control	23·1	
13.	N/40	Aqueous	1 min. at 100° C.	0·4	
		"	Control	11·7	
		Alcohol	1 min. at 100° C,	14·0	
		"	Control	21·5	

These and several other experiments show that the alcoholic extracts retain very much more of their power to digest fibrin after being heated than do the corresponding aqueous extracts. The amount of protein and other nitrogenous substances was practically the same in the two sets of extracts.

It might be supposed that the alcohol itself in some way protected the trypsin from destruction when heated, or that the alcoholic extracts did not reach so high a temperature when heated, owing to the lower boiling point of alcohol compared with water. To test this, corresponding aqueous and alcoholic extracts were diluted with alcohol and water respectively, so that the percentage of alcohol was the same in both. The diluted extracts were then heated to exactly the same extent in a water bath and their digestive powers tested on fibrin.

No.	Reaction (HCl)	Extract	Treatment	Fibrin digested in cc. of N/10 nitrogen.
14.	N/40	Alcohol (20%)	1 min. at 100° C.	21·1
		"	Control	23·1
		Aqueous + alcohol	1 min. at 100° C.	0·0
		"	Control	18·9
15.	N/40	Alcohol	1 min. at 100° C.	14·0
		"	Control	21·5
		Aqueous + alcohol	1 min. at 100° C.	0·0
		"	Control	7·9

The addition of alcohol to an aqueous extract of pancreas does not afford any protection against heat as far as the action of the trypsin on fibrin is concerned.

In the original experiments on the resistance of trypsin to heat the solutions, when heated, contained only a very small amount of nitrogen, not more in some cases than 0·02 per cent. The pancreatic extracts used in the experiments now described were very much richer in nitrogen and generally contained 15 to 20 times as much as the older extracts. This corresponds to a considerable amount of protein in the solution, and as protein is known to form a loose compound with hydrochloric acid under some conditions, it was resolved to try the effect of considerably higher amounts of acid in the solutions to be heated.

It was now found that the protected afforded to trypsin solutions when heated depends on the hydrogen ion concentration, and the more protein there is present the more hydrochloric acid must be added to protect the trypsin from destruction when heated. The following experiments show this increasing protection with increased amount of acid.

No.	Reaction (HCl)	Treatment	Fibrin digested cc. N/10 nitrogen	Caseinogen digested cc. N/10 nitrogen
16.	N/50	1 min. at 100° C.	5·6	21·1
		Control	16·9	39·1
17.	N/23	1 min. at 100° C.		25·1
		Control		35·7
18.	N/17	1 min. at 100° C.	7·4	26·8
		Control	7·6	26·7
19.	N/15	1 min. at 100° C.	10·9	26·2
		Control	10·9	26·4

The same specimen of trypsin was used in these experiments.

No.	Reaction (HCl)	Treatment	Fibrin digested cc. N/10 nitrogen	Caseinogen digested cc. N/10 nitrogen
20.	N/15	2 min. at 100° C.	3·5	19·2
		Control	11·6	28·7
21.	N/12	2 min. at 100° C.	8·4	14·8
		Control	12·2	15·6

The same trypsin solution was used in these two experiments. Similar results have been obtained with many other aqueous extracts of pancreas prepared in the laboratory, and it may be stated generally that the higher the proportion of nitrogen in the extract, the greater the amount of hydrochloric acid which must be added in order to prevent destruction of the trypsin by heat. In a few cases, for example experiments 18 and 19, it was found that the protection against heat afforded by a given amount of hydrochloric acid was the same as regards both digestion of fibrin and of caseinogen. Usually, however, the destruction of the fibrin digesting power was considerably greater than that of the caseinogen digesting power.

It was pointed out by Mellanby and Woolley (3) that in acid of the strength of N/20 HCl trypsin is slowly destroyed at 16° C. and more rapidly at 35° C. Apparently at room temperature about half of the trypsin is destroyed in four hours, and two thirds in a day. The activity of the trypsin in their experiments was estimated by the power to coagulate calcified milk.

Other references to the effect of hydrochloric acid on trypsin at moderate temperatures have already been mentioned (2).

It was also found by Lénard (4) that if trypsin were rendered inactive by addition of acid, only a trace of its activity is

restored by neutralising and then adding alkali. Long and Johnson (5) state that 0·3 per cent hydrochloric acid has no effect on trypsin in the course of incubation for half an hour. These observers appear to have tested the activity of the trypsin on one substrate only, but in the following experiments the action of hydrochloric acid at room or body temperature on trypsin has been tested as regards the power to digest both fibrin and caseinogen.

Alcoholic (15 per cent) extracts of pig's pancreas were used. In each case 1 cc. of the original trypsin was compared with that quantity of the trypsin + acid which would contain 1 cc. of trypsin originally, and the solutions adjusted so as to contain the same amount of sodium chloride. Digestion both of fibrin and caseinogen was carried on in presence of 0·5 per cent sodium carbonate, 1 g. fibrin or 0·6 g. caseinogen being used, in about 40 cc. of fluid. Digestion as usual is expressed in cc. of N/10 nitrogen, the estimation being done as already described.

22. 10 cc. trypsin + 20 cc. N HCl. Kept at 36° C. for 12 min.
20 cc. N NaOH then added.

Digestion by control	21·6 cc.	fibrin,	38·2 cc.	caseinogen
" " treated trypsin	0·0 cc.	"	8·8 cc.	"

23. 20 cc. trypsin + 10 cc. N HCl. Kept at 16° C. for 4 days.
10 cc. N NaOH then added.

Digestion by control	12·1 cc.	fibrin,	31·6 cc.	caseinogen
" " treated trypsin	0·0 cc.	"	5·4 cc.	"

24. 40 cc. trypsin + 20 cc. N HCl. Kept at room temperature for 11 days. 20 cc. N NaOH then added.

Digestion by control 11·7 cc. fibrin, 38·7 cc. caseinogen

" " treated trypsin 0·0 cc. " 5·6 cc. "

It will be seen from these experiments that the power of trypsin to digest fibrin is destroyed considerably more readily in acid solution at moderate temperatures than is the power to digest caseinogen. It is also seen that the power to digest caseinogen withstands a considerably higher percentage of hydrochloric acid than has been generally supposed, the strength of acid being N/3 in these and several other experiments with similar results. In one experiment, after 24 hours at room temperature in N/3 hydrochloric acid, the trypsin still retained about 10 per cent of its original fibrin digesting power, but otherwise no fibrin was digested at all by trypsin after treatment with acid of this strength for a day or upwards.

On the whole, then, treatment of trypsin solutions with hydrochloric acid either at high or low temperatures shows that the power to digest fibrin is considerably more readily destroyed than the power to digest caseinogen. This bears out the theory discussed previously (1) that in some ways the fibrin digesting power is more sensitive to outside influences and again points to the hydrolysis of fibrin and of caseinogen being carried out by different groups or side chains, those digesting caseinogen being more stable than the others.

In an earlier paper (1) the work of Fermi was referred to as showing that after treatment with various reagents trypsin would no longer digest fibrin but would still digest gelatin. Pollak was also mentioned as finding that with different enzyme preparations the relative amounts of serum and gelatin varied enormously. This was ascribed by Pollak to the presence of a "glutinase" which acted only on gelatin.

I have found that the relative amount of fibrin and caseinogen digested by different trypsin solutions also varies very much, and this without subjecting the enzyme to treatment of any kind. Thus, three enzyme solutions prepared in exactly the same way, by extracting minced pancreas (sheep) with three times its weight of water for 14 days and filtering were taken. To 1 cc. of each were added 40 cc. of 0·5% Na_2CO_3 and 1 g. fibrin on the one hand, and 40 cc. of 1·5% caseinogen in 0·5% Na_2CO_3 on the other hand. Digestion of fibrin lasted 2 hours, and of caseinogen 1 hour. Amount of digestion stated in cc. of N/10 nitrogen, estimated in the usual way.

Enzyme	Fibrin digested	Caseinogen digested
1.	12·4 cc.	33·7
2.	16·1 cc.	32·9
3.	3·3 cc.	17·6

The difference between Nos. 2 and 3 is very marked.

When pancreatic extracts were kept for a considerable time, it was found that the fibrin digesting power as a rule diminished to a very much greater extent than the caseinogen digesting power. In one extreme case I examined a solution of trypsin

which had been kept in the laboratory for over 10 years. It had no digestive action on fibrin whatever, but still digested caseinogen to the extent of 26·6 cc. under the above conditions. These facts afford further evidence that the digestion of fibrin and of caseinogen by pancreatic extracts are either due to different enzymes or at least to different groups of the molecule if only one enzyme is concerned.

In my previous paper (2) I mentioned that the power to digest caseinogen seemed to be less affected by heat than the power to coagulate milk, which was taken as the measure of the activity of trypsin by Mellanby and Woolley. I further suggested that these two functions might be due to different sets of side chains. In a later paper Mellanby and Woolley (6) take exception to my suggestion and say "Pancreatic rennin and trypsin are identical. In fact the coagulation of milk by trypsin is an expression of a general law that all proteolytic ferments coagulate milk provided sufficient Calcium be contained in it". They further say "The unique fact that the ferment or ferments in pancreatic juice which digest protein and coagulate milk should withstand boiling in acid solution is practically conclusive proof that the two actions are produced by one and the same substance".

If this assumption of Mellanby and Woolley is correct, however, then the milk coagulating power and the hydrolytic power of pancreatic extracts should presumably be quite parallel in their behaviour. The experiments detailed in the present paper and those in a previous paper (1), however, tend to show that

the digestion of fibrin and caseinogen if carried out by one enzyme at least involve two sets of groups of the enzyme molecule, and therefore cannot really be said to be produced by the same substance in the sense evidently meant by Mellanby and Woolley.

I have also carried out some experiments comparing the milk coagulating power of pancreatic extracts with their proteolytic power and shall now deal with these.

25. To 25 cc. pancreatic extract was added 0·5 cc. N HCl. Half of this was then heated to 100° C. for 1 minute and filtered

	Digestion in cc.
a. 1 cc. trypsin, 40 cc. 0·5% Na_2CO_3	21·0
b. 1 cc. trypsin (heated) 40 cc. 0·5% Na_2CO_3	10·2

1 g. fibrin. Digestion 2·75 hours.

To 20 cc. milk was added 1 cc. trypsin (1) fresh trypsin
(2) heated "

(1) Complete coagulation in 6 minutes (at 37° C.)

(2) No coagulation in 3 hours. "

26. Similar to the last experiment.

	Digestion in cc.
a. 1 cc. trypsin, 40 cc. 0·5% Na_2CO_3	26·2
b. 1 cc. trypsin (heated) 40 cc. 0·5% Na_2CO_3	8·1

(1) Complete coagulation in 5 minutes.

(2) No coagulation in 2 hours.

From these two experiments it will be seen that though the heated extract is still able to digest a considerable amount of fibrin, its milk coagulating power, if any, is negligible.

27. To 20 cc. extract was added 0·5 cc. N HCl. Half was heated to 100° C. for 1 minute and filtered.

	Digestion in cc.
a. 1 cc. trypsin, 20 cc. milk	39·3
b. 1 cc. trypsin (heated) 20 cc. milk	4·6

Digestion 1 hour. Amount estimated by tannic acid.

To 20 cc. milk added (1) 1 cc. fresh trypsin

(2) 1 cc. heated trypsin

(1) Complete coagulation in 5 minutes (at 37° C.)

(2) No coagulation in 2 hours. "

28. Similar to above.

	Digestion in cc.
a. 1 cc. trypsin, 20 cc. milk	38·4
b. 1 cc. trypsin (heated) 20 cc. milk	3·9

(1) Complete coagulation in 5 minutes.

(2) No coagulation in 2 hours.

These experiments confirm Nos. 25 and 26 in showing that the milk coagulating power of pancreatic extracts is more readily destroyed by heat than the proteolytic power.

More striking differences are found between the milk coagulating power and the proteolytic action of pancreatic extracts under certain conditions without subjecting these to any such drastic treatment as heating to 100° C. involves.

Edkins (7) found that fresh, active extracts of pancreas were not so good at altering milk and producing Roberts' metacasein reaction as older extracts, but that the proteolytic action was greater in the fresh extracts. He suggested the metacasein

reaction as being an aspect of the proteolytic enzyme of the pancreas. Halliburton and Brodie (8) confirmed what had been pointed out by Benger, that freshly prepared extract of pig's pancreas had very little curdling action on milk, but acquired this property on being kept for a considerable time. They accounted for this fact by supposing that the trypsin at first masks or hinders the milk curdling enzyme, but that the former enzyme deteriorates more quickly and so finally allows the rennin to reveal its presence. Vernon (9) found similar results and stated that the ratio of rennin value to tryptic value varied largely in different extracts of pancreas and also in the same extract at different times. In alcoholic extracts the ratio usually got higher as the extract got older, the tryptic value deteriorating more rapidly than the rennin. In glycerol extracts, however, the ratio got less after say nine weeks, the trypsin being liberated more slowly from its zymogen than the rennin. Some glycerol extracts which were very rich in trypsin gave practically no clot at all, as though the clot was dissolved nearly as fast as it was formed. In a later paper Vernon (10) found that rennin and trypsin were precipitated from pancreatic extracts to practically the same extent when excess of alcohol was added. He considered that in the case of trypsin some groups have the power of coagulating milk and others have the proteolytic power.

I have tested a number of pancreatic extracts at different stages and record some of the principal results below.

In each case sheep's pancreas was used. It was minced and extracted with 2·5 times its weight of water and a little chloroform. The experiments were similar to Nos. 25-28 in technique. In all cases, as usual, chloroform was added to exclude bacterial action when proteolytic power was being tested.

29. Pancreas extracted two days, then filtered.

	Digestion in cc.
1 cc. trypsin, 40 cc. 0·5% Na_2CO_3	14·9
1 g. fibrin. Digestion 3 hours.	
1 cc. trypsin, 40 cc. caseinogen	44·6
Digestion 1 hour.	
1 cc. trypsin, 40 cc. milk	23·3
Digestion 0·5 hour.	

1 cc. extract + 20 cc. milk. No coagulation in 0·5 hour. No coagulation on adding an active coagulating extract at the end of this period, the caseinogen having now been digested into products which no longer give a coagulum.

30. Pancreas extracted 1·5 hours, then filtered.

Fibrin digested in 3 hours, 26·4 cc.

Caseinogen digested (1 hour) 41·8 cc.

No coagulation of milk in 0·5 hour, but much digestion as shown by the small precipitate on adding tannic acid and the fact that an active extract now fails to produce coagulation.

31. Pancreas extracted 3 days, then filtered.

Milk digested in 0·5 hour, 26·7 cc.

No coagulation of milk.

32. Pancreas extracted 3 days, then filtered.

Milk digested in 0·5 hour, 31·0 cc.

No coagulation of milk.

From these experiments we see that freshly prepared extracts of pancreas generally do not coagulate milk but are very active proteolytic agents, both on fibrin and on caseinogen.

In a few cases I have found that the filtrate after three hours extraction coagulated milk rapidly, but this was exceptional.

It has already been mentioned that Halliburton and Brodie accounted for similar facts by supposing that the trypsin deteriorates more quickly and in a short time allows the rennin to reveal its presence. Vernon appears to suggest that a clot may be formed but gets redissolved almost at once.

In the extracts employed by me the rennin seemed to be fully developed in 15 days or less, but I have found no appreciable diminution of the proteolytic power within that time.

The extract used in experiment 30 was tested finally when five weeks old. Coagulating power had been noticed considerably before this, however. Milk was now coagulated in four minutes, and the amount of caseinogen digested was now 44·7 cc. this being actually more than it had digested at first. If in the first case a clot was formed but almost immediately redissolved this should have been still more so when the proteolytic power had increased.

In other cases the rennin seemed to have developed completely within four days, the proteolytic power being practically the same as at first, and there was no evidence of any clot having been formed at all when the extract was first tested.

These last experiments once more show that great differences exist in pancreatic extracts in respect of their proteolytic and milk coagulating powers. This again points to there being either two or more enzymes present, which develop from their precursors at very different rates, or at least the groups which are responsible for the different functions develop their properties quite independently.

SUMMARY.

The amount of acid required in order to protect trypsin from destruction by heat depends on the amount of protein present. The more protein in solution, the more acid is required.

If not enough acid is present to afford complete protection to the trypsin, the fibrin digesting power is usually destroyed by heat to a considerably greater extent than the power to digest caseinogen.

Hydrochloric acid at moderate temperatures also destroys the fibrin digesting power considerably more rapidly than the caseinogen digesting power.

The relative amounts of fibrin and caseinogen digested varies very much in different pancreatic extracts.

The milk coagulating power is more easily destroyed by heat than the proteolytic power.

Generally, but not always, freshly prepared pancreatic extracts have no milk coagulating power. Such extracts are always actively proteolytic, but the proteolytic power does not fall off so rapidly as to justify the assumption that the non-appearance of a coagulum with milk is due to the coagulum being really formed but instantly redissolved.

All these facts point to the proteolytic and milk coagulating functions of pancreatic extracts being due to a number of distinct enzymes, or, if only one enzyme is present, the various actions are brought about by different groups of the molecule.

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A NOTE ON THE QUESTION OF THE IDENTITY OF PEPSIN AND RENNIN.

Much has been written on the question of the identity of pepsin and gastric rennin, and two theories have been brought forward. One, first suggested by Pavlov (1) is that pepsin and rennin are identical. Savjalov (2) and Gewin (3) who supported this view held that coagulation is the first in the digestion of milk by pepsin. The identity theory is based on the parallelism between the behaviour of the proteolytic and milk coagulating actions of gastric extracts under different conditions. The other theory is that the enzymes are different. There are two possibilities here, one, put forward by Nencki and Sieber (4) and others being that "pepsin" consists of a large molecule with different side chains, one set of which digests protein in acid solution, while another set coagulates milk in neutral solution.

A second possibility is that there are two distinct enzymes involved in the two functions. This is the theory which has been mainly developed by Hammarsten (5). It is difficult to distinguish experimentally between these two possibilities, and indeed the present state of our knowledge of the constitution of enzymes renders a distinction hardly possible. Both depend on the fact that by suitable treatment the two actions can be separated from one another, so that a solution may be obtained which coagulates milk but has no proteolytic action, while on the other hand it is possible to prepare an active proteolytic solution which will not coagulate milk.

Porter (6) in support of Hammarsten's theory found that various commercial preparations, while actively milk coagulating, were anti- peptic.

I do not intend to deal further with the literature of the subject in general in this note. A full discussion of the question is given by Oppenheimer (7).

In a research on the development of enzymes from foetal life onwards I tested the properties of extracts of stomachs of young and adult rabbits, and found the differences stated here. According to Oppenheimer pepsin is already present in the stomach of rabbits before birth, while Gmelin (8) found rennin to be absent from the stomach of new born animals. Others have found that rennin develops very rapidly after birth.

Rakoczy (9) and van Hasselt (10) found that in the case of the ox the rennin disappeared rapidly during the first month after birth, while the pepsin rapidly increased, but a calf nine days old was apparently the youngest animal to be employed by Rakoczy while van Hasselt used stomachs of "nüchternen" animals, by which is evidently meant animals being suckled or quite young.

In my experiments the stomachs of rabbits were taken as soon as possible after birth, washed out thoroughly with water and ground up with twice their weight of water. A little chloroform was added and after three days extraction the liquid was filtered off and tested. Extracts of stomachs of adult rabbits were prepared in exactly the same way. The extracts were subjected to no treatment whatever with acid or other reagents used by Hammarsten and others with a view to destroying the proteolytic or milk coagulating function as the case might be.

In the coagulation experiments 1 cc. extract + 5 cc. milk were kept at 37° C. and examined every five minutes. Coagulation was

considered complete when the test tube could be inverted without disturbance of the contents. Experiments were always repeated at least three times.

The proteolytic experiments were carried out on ox fibrin which had been minced, washed and heated to 85° C. 1 cc. extract + 1 g. fibrin + 40 cc. N/20 HCl were kept at 37° C. for a certain time and then filtered, the nitrogen in the filtrate being estimated by Kjeldahl's method. Controls with fibrin and acid alone were always carried out (the acid was found never to dissolve any fibrin) and allowance was made for the nitrogen in the extracts.

The following are the results so far obtained:-

New born rabbits. Each represents a separate litter.

No.	Fibrin digested in 2 hours. (in cc. of N/10 nitrogen)	Coagulation time.
1.	0·0	12 minutes.
2.	0·0	18 "
3.	0·0	30 "
4.	0·0	12 "
5.	0·0	13 "
6.	0·0	22 "

Adult rabbits.

No.	Fibrin digested in 1 hour.	Coagulation time.
1.	4·6	No coagulation in 2 hours.
2.	8·0	" " 2 "
3.	9·3	" " 1 "
4.	23·8	" " 2 "
5.	8·4	" " 2 "

These results show the very wide differences which exist between extracts of stomachs of young and adult rabbits in regard to the content of rennin and pepsin.

In some cases the extract of young rabbit stomach was incubated with fibrin and acid for 18 hours without any appreciable digestion taking place.

At the end of every coagulation experiment in the case of the adult extracts, a few drops of active rennin were added to the mixture of extract and milk. Coagulation now always took place very rapidly, proving, if need be, that the absence of coagulation at first was not due to any abnormality in the milk, but simply to lack of rennin in the extract.

These facts, so far as they go, seem to argue against pepsin and rennin being identical. The two sets of extracts were exactly similar in mode of preparation and it is unlikely that one set would contain any inhibitory substance which would be absent from the other.

In no case did I find any extract to give a result different from those described. The extract from young rabbits never failed to give a perfectly firm clot within 30 minutes, while the adult extract never showed the least sign of being able to coagulate milk.

No extract from young rabbits, on the other hand, caused the slightest digestion of fibrin, but the adult extracts always had this proteolytic action.

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